FORMATION, MELTING AND INTERACTION OF POLYSACCHARIDE HELICES

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ABSTRACT

A brief review of ordered polysaccharide conformations is presented and the importance of such conformations is illustrated by reference to another family of biopolymers, proteins.

The double helix-random coil transition of carrageenan and its involvement in the formation of thermally reversible gels has been studied, mainly by optical rotation. Chemical degradation at specific sugar residues has been used to produce a "segmented" kappa-carrageenan which undergoes the helix-coil transition without gel formation. This product has been used to study the transition without interference from gel formation. The evidence presented suggests that the hysteresis observed in gel melting and setting arises from aggregation of the double helices.

The effect of degree of sulphation of the carrageenans was examined and a possible biological role is suggested for the relation between aggregation of the gel network, the physical properties of the gel and the extent of sulphation of the polymer.

An interaction between the ordered kappa-carrageenan conformation and certain seed galactomannans, e.g. locust bean gum, is described both for the native carrageenan polymer and the segmented product. A model for this system, which shows ligand induction of polysaccharide conformation changes, is suggested. This interaction can be regarded as a "polysaccharide quaternary structure". An analogy is drawn with the interaction of matrix and skeletal polysaccharides in the plant cell wall.

A series of carbohydrate polymers has been surveyed, by optical rotation, for thermally reversible conformational transitions. These studies indicate that carrageenan-like transitions are rare in polysaccharides.

An attempt has been made to explain the optical rotation anomaly of laminaran and to substantiate the triple helix model proposed for β-1,3-glucan gels and networks.

Part of the work described in Chapter II of this thesis has been published in collaboration with Dr. I.C.M. Dea and Prof. D.A. Rees. A reprint of this publication is included at the end of the thesis.

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INTRODUCTION

In the early 1950's a major revolution in biological science was sparked off by the derivation of chain conformations for two naturally occurring polymers. These were the α - helix of some protein chains[1] and the double helix of DNA.[2] Since then a vast amount of scientific effort has been directed toward the conformation of biopolymers in the belief that a knowledge of such properties will explain the "mysteries" of these molecules. In fact, over the last twenty years, many of the functions of nucleic acids and proteins have been rationalised in terms of their shape and structure. The potential in continuing this approach is obvious, perhaps especially in a third type of biopolymer, polysaccharides. In recent years some significant advances in the relation between the shapes of some polysaccharides and their functions have been reported.[3]

The techniques developed and used in protein and nucleic acid chemistry can often be applied to polysaccharide systems. However, the width of this subject makes a sweeping review impractical and it may be of more interest and of more relevance to later discussion in this thesis to look closely at two areas of protein chemistry where structure, from molecular organisation down to the level of individual atoms, has been related to the function of the molecule.

A. Ligand Induced Changes of Enzyme Conformation

Although the structure of several enzymes has now been elucidated[4] this has not automatically explained some aspects of enzyme behaviour which have puzzled biochemists since their discovery,

i.e. their remarkable specificity and catalytic power. Early theories stemmed from the ideas of Emil Fischer[5] who postulated that the substrate and enzyme interlock like the pieces of a jigsaw, catalysis being facilitated by the correct proximity and orientation of the catalytic group and the bonds of the substrate involved, and specificity being facilitated by a unique fit of the shape of the substrate to a "hole" in the enzyme particle.

These ideas have stood the test of time and can now be expressed in molecular terms, for example in the induced fit theory which Koshland[6] has proposed for some enzyme substrate complexes. Here the substrate must not only fit specifically to the enzyme but it must also induce the catalytic group of the enzyme to move into the correct position for reaction. This ligand induced conformation change has received support from many examples, the most striking from carboxypeptidase[7] where a tyrosine in the active site moves by as much as 15 A^{o}.

More recently a third puzzle about enzymes has arisen from the fact that small molecules which are not involved in catalysis can still affect the velocity of enzyme activity.[8] This allows the regulation of various biological processes, e.g. feedback inhibition, where the product of a reaction sequence inactivates an earlier enzyme in the sequence. The basis of these effects was soon recognised to stem from the subunit structure of regulatory proteins and the key to molecular models of these systems came from the fact that they exhibit co-operative effects.[9]

Monod and co-workers[10] proposed that these proteins have sites, called allosteric sites, which bind inhibitor. These sites

are removed from the active site but can exert control over it. The model of Monod, Wyman and Changeux proposed:[11]

a) the protein subunits are arranged in a symmetrical manner;

b) change in the conformation of one subunit is followed by an identical change in all the other subunits (thus preserving the symmetry);

c) conformational changes occur between at least two states, one of which, present in small amounts, binds substrate preferentially. Thus the addition of substrate will increase the fraction of this state present so leading to co-operativity.

Regulation by other molecules is explained by a similar argument if an activator, acting at an allosteric site, is preferentially bound to the state which binds substrate and inhibitor is bound to a state which does not. Koshland and co-workers[12] have indicated how their theory of ligand induction can be extended to allosteric proteins. They argue that when ligand (substrate, activator or inhibitor) binds to one subunit its shape may change. This deformation could affect the interaction with and hence the shape of neighbouring subunits, thus influencing the reaction of this subunit with substrate. These changes are seen as occurring sequentially, i.e. enzyme symmetry is not necessarily maintained. Such reasoning, put on a quantitative basis, has been successfully applied to the enzyme-like kinetics observed for haemoglobin.[9] Several experiments have tested the relative validity of the two theories[9], perhaps the most interesting on haemoglobin derivatives where two of the four subunits have been

locked in the conformation which has high affinity for substrate.[13] This is achieved by converting either the two α or the two β subunits of haemoglobin to the cyanomet which has the same conformation as the subunits of the oxygenated molecule. The addition of oxygen to the first of these derivatives is illustrated schematically in fig. 0.1.

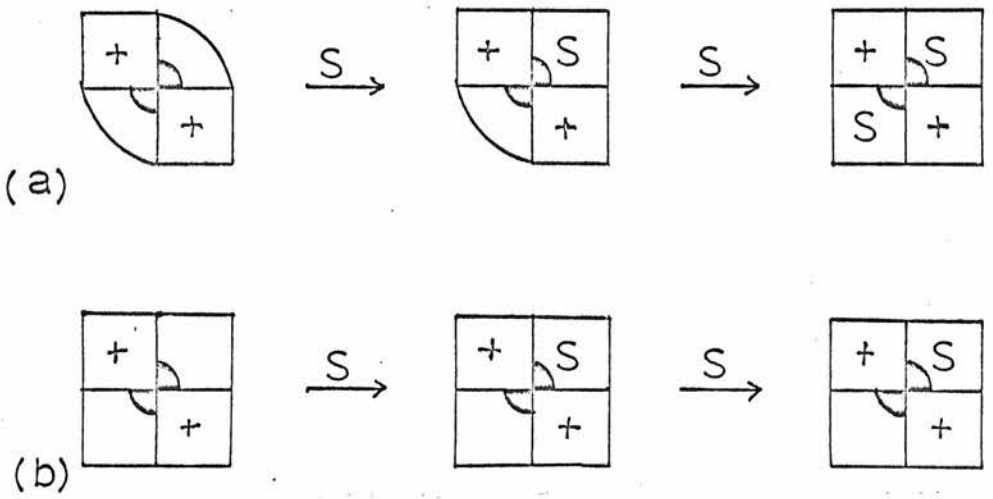


Fig. 0.1. Illustration of the addition of oxygen to a tetrameric protein with the α subunits oxidised (+). If the Koshland model (a) is obeyed binding of oxygen involves the same α – β subunit changes in each step but the β – β interactions are different. If the concerted model of Monod (b) is obeyed neither the α – β nor the β – β subunit changes will alter (from ref. 12).

If the symmetry model of Monod is followed not only the two subunits which have been oxidised to the cyanomet but also the two which have not will be in the conformation that preferentially binds oxygen. The addition of one further ligand (step 1) will not change the conformation of the subunit to which it binds; thus

the conformation of the remaining subunit will not be affected by this step and the reaction will not show co-operativity. If co-operativity is observed it must indicate that binding of ligand to one subunit affects the other subunit. Co-operativity, and hence interaction, is in fact observed between β subunits and the fact that the half oxygenated hybrids are saturated at much lower oxygen concentrations than normal haemoglobin shows the importance of α - β subunit interaction.

In 1962, Koshland[14] calculated that effects of proximity and orientation alone could not account for the observed magnitude of difference between enzymes and other catalysts, thus he and Storm[15] were led to propose that ligand induced changes actually align the electronic orbitals of the reacting atoms along the ideal path for the reaction. Support for this theory has come from a study of the rate of esterification with ethanol of various carboxylic acids where the angle of approach of the reacting atom is dictated by steric hindrances in the acid molecule. In certain cases vastly increased reaction rate (10^6 times) were observed.[9] Further evidence was obtained by converting a serine residue in the active site of the enzyme subtilisin to a cysteine residue (R-OH to R-SH)[16]. The enzyme, which was otherwise unchanged, was now found to be completely inactive. In non-enzymic reactions of the same type the reactivity of the sulphur and oxygen analogues would be similar, so they reason that, as the distribution of orbitals in oxygen and sulphur differ, the necessary alignment of orbitals is no longer attained.

Orbital steering will also account for enzyme specificity, as if such exact alignment is required, then any slight deviation will

greatly affect the reaction rate. Similar arguments can be applied to regulatory control as a change bringing about alignment will activate an enzyme and one destroying alignment will inhibit an enzyme.[9] Despite the obvious attraction of orbital steering it is worth noting that the idea is not accepted by all workers in the field[4].

B. Collagen Structure and Function

The α - helix, which is a common component of the structure of globular proteins,[4] retains its importance in fibrous proteins such as keratin, myosin, tropomyosin and paramyosin where the molecule exists as a coiled coil, generally of two strands.[17] Perhaps the closest analogy between the structure of amino acid polymers and carbohydrate polymers is between collagen and carrageenan or β-1,3-glucan, two of the polysaccharides studied in this thesis. It is perhaps no coincidence that these three biopolymers also serve a roughly similar function. Collagen, which occurs in many tissues of all multicellular living species, is the most abundant mammalian protein. Its principal functions are the provision of mechanical stability in an extracellular framework (e.g. in bone) and the provision of high tensile strength (e.g. in tendon). It furnishes an interesting example of how the properties necessary to perform such functions are derived from the primary, secondary and tertiary structure of a macromolecule.

Tropocollagen consists of three continuous parallel peptide chains of approximately 1000 units[18] combined in a twisted triple helix.[19] (fig. 0.2)

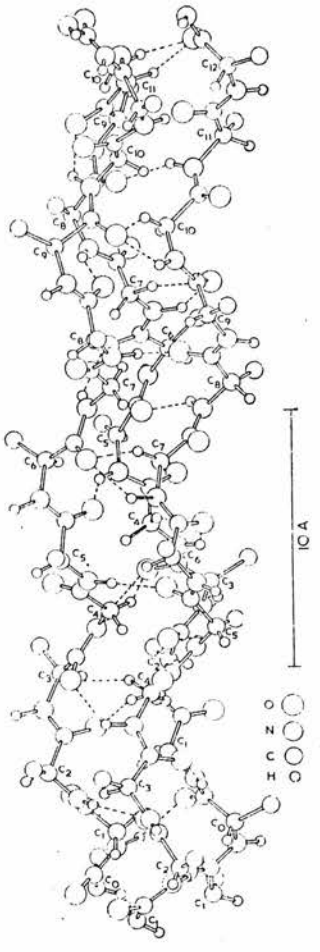


Fig. 0.2. The collagen triple helix (from ref. 19).

Amino acid analysis and sequencing of the chains have shown collagen to be quite unique among proteins in that it has a high content of proline and hydroxyproline and over almost the entire length every third residue is glycine.[20]

Although collagen chains have this periodic sequence of glycine residues, and would thus be expected to adopt a periodic conformation, the common polypeptide conformations of this type are not possible as proline and hydroxyproline are unable to participate in an α - helix and only with difficulty can they be induced to take part in a β - pleated sheet conformation. Homopolymers of these

residues, however can form helical structures [21 a,b] and it is a conformation like the polyproline II helix which is adopted in collagen. The high content of proline and hydroxyproline have been recognised for some time as one of the major stabilising factors of the triple helix.[22] It has in fact been shown that the variation in the thermal stability of vertebrate collagen with the environment temperature of the species[23] bears a linear relationship to the imino acid content of the collagen.[24] This variation is necessary as the collagen must be stable enough to perform its function without being so stable that it cannot be turned over in living tissue. The geometry of the triple helix demands that the α-carbon of every third amino acid residue is situated in the centre of the molecule. Thus glycine, which is the only amino acid with no side group on the α-carbon, is the ideal in terms of steric interactions.[19] It is now known that both the N-terminal and C-terminal regions have less than one third of their residues glycine, thus they are not helical but appear to be important in crosslinking and antigenicity.[20]

The stability of the triple helix is based on interchain hydrogen bonding[19] but the structure is further strengthened by strong covalent crosslinking between the chains. These cross links are formed extracellularly by enzymic oxidative deamination of lysine and hydroxylysine residues to allysine and hydroxyallysine which then cross link spontaneously by various condensation reactions[20]. (fig. 0.3)

The rods of tropocollagen are laid together in parallel to form fibrils up to 2000 A^{o} in diameter. Then, according to the use to which they are to be put, further aggregation of these fibrils takes

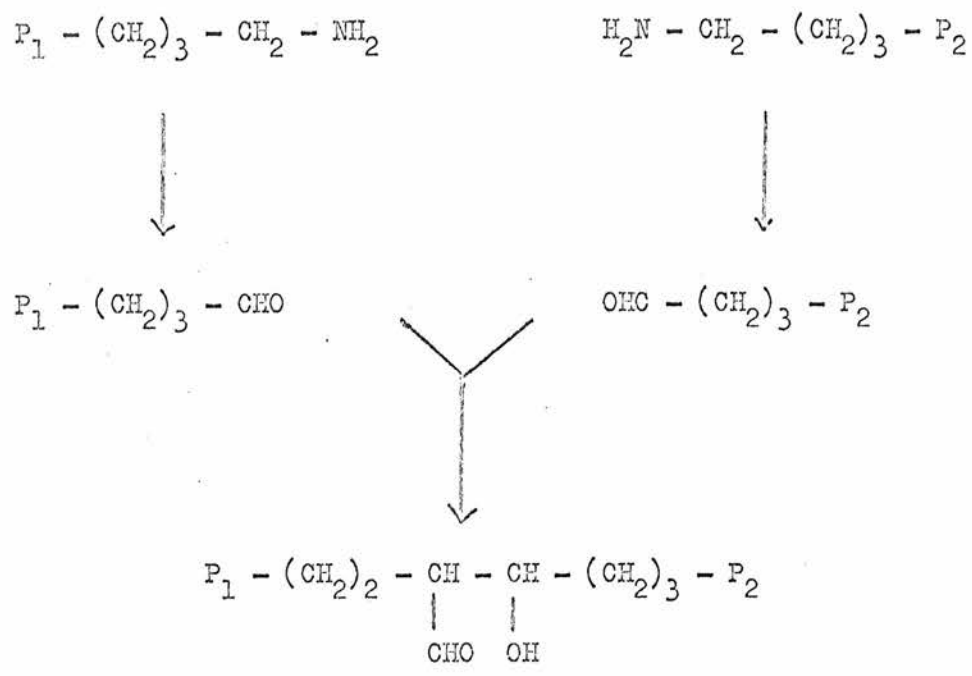


Fig. 0.3. Example of crosslinking proposed for collagen. This aldol condensation leads to an "allysine aldol". Aldimine condensations also take place (lysine and hydroxylysine with their aldehydes). All possible combinations of these condensations may be products.

place. The arrangement of the molecules is, as yet, not fully elucidated but several models have been proposed on the basis of.

a) Lateral order shown by low angle X-ray photographs of rat tail tendon collagen.[25]

b) Reaggregation from tropocollagen solutions.

c) Electron microscopy of a particular type of these aggregates [viz. "segment long spacing" (S.L.S.) aggregates] which show a molecular length of roughly four times the native fibre repeat (D).

Observation of these patterns led Schmitt et al [26] to propose the "quarter stagger model". However closer examination of the patterns has shown the factor to be 4.4 D and from this Hodge and co-workers [27] have postulated the model shown in fig. 0.4 (a). Veis et al [28] have based a model on microfibrils composed of tetramers of tropocollagen molecules. This model (fig. 0.4 (b)) gives regularly spaced regions of holes and overlaps although the molecules are spaced in three intervals of D followed by one of 2D. A third model by Smith [29] is shown in fig. 0.4 (c). Five chains of tropocollagen in the modified quarter stagger arrangement of Hodge and Petruska are built up into filaments which are staggered by multiples of D in the fibril.

The low angle X-ray evidence has been interpreted in three ways none of which appears to have more merit than the others. [20] All three models contain about 150 molecules in cross section and are the cylindrical lattice, the spiral roll structure and the limited hexagon lattice [fig. 0.4 (d)]. Burge [30] has suggested that all the X-ray data can be explained only by invoking a pair of molecules twisted about each other to form an extended double helix.

These aggregates derive a large measure of their stability from intermolecular cross linking of the same type as the intramolecular linking mentioned above.

Organisation of the fibrils to form the fibre of a particular tissue depends on the role collagen is to perform in that tissue. In tendon, for example, the fibrils aggregate in parallel giving a structure which will transmit tension. The fibres also

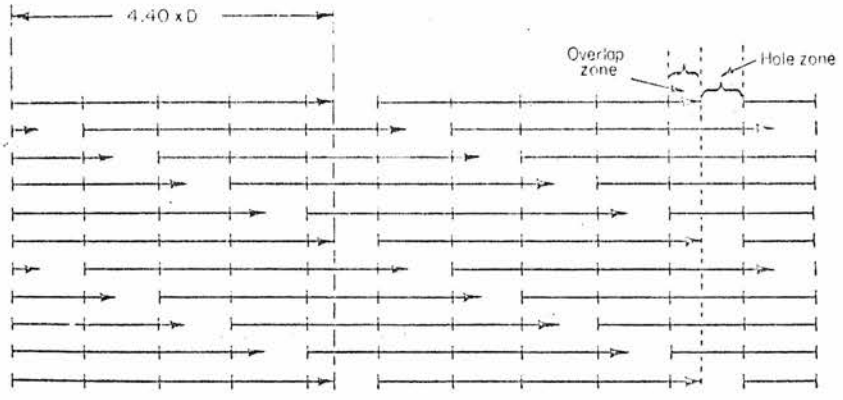


Fig. 0.4(a) Proposed packing in native collagen fibrils showing overlap and hole zones. (Hodge, 1967 [27]).

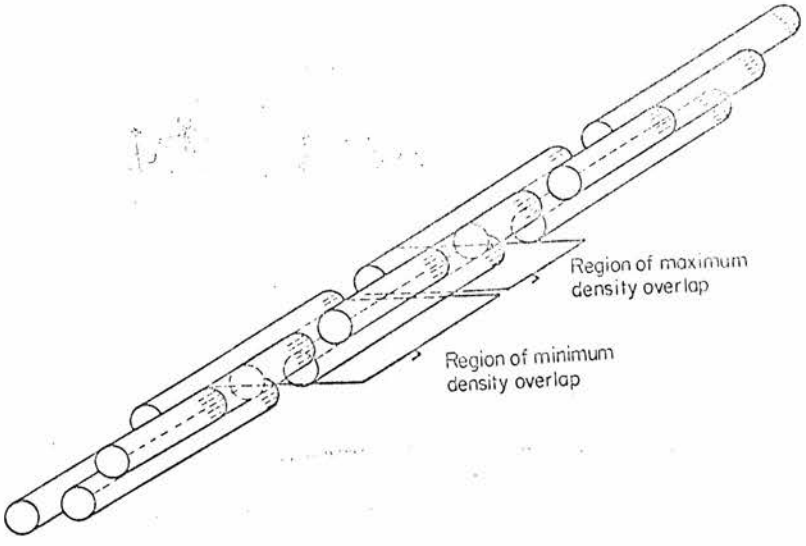


Fig. 0.4(b) Proposed packing of tetramolecular units to form microfibrils. (Veis et al, 1967 [28]).

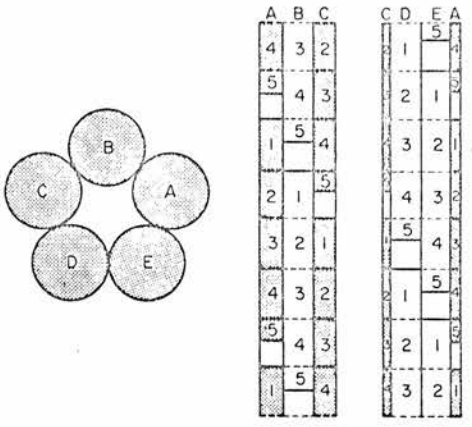


Fig. 0.4(c) Proposed structure of filament. (i) cross-section, (ii) perpendicular to its length from two directions. (Smith, 1968 [29])

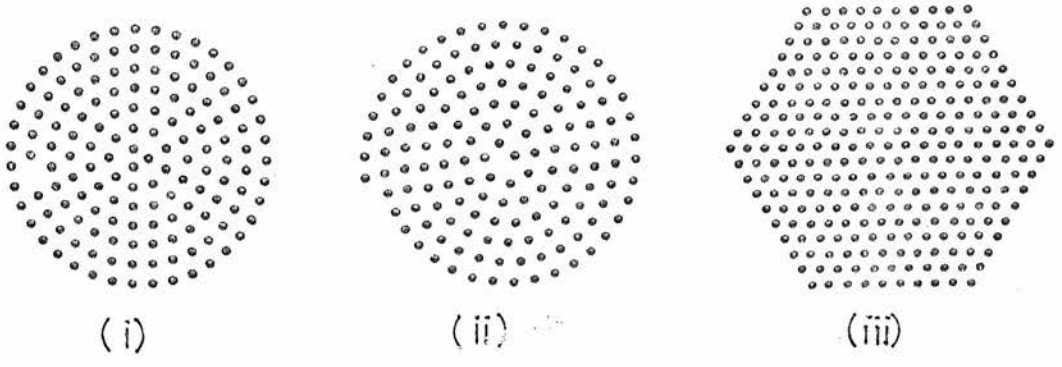


Fig. 0.4(d). Cross-sections of cylindrical (i) spiral (ii) and hexagonal (iii) lattices proposed for collagen fibrils from low angle X-ray diffraction patterns.

occur in laminated sheets in such places as the cornea of the eye where a tough sheath is required, while in intercellular matrices, e.g. Wharton's Jelly, they occur as a random network of thin strands [31].

It is of particular interest in this thesis to see the parallel between solution studies on collagen and carrageenan, some aspects of which are discussed in Chapter I.

C. Nucleic Acids

Nucleic acids have a vital function in cellular reproduction as it is these molecules which transmit the genetic information necessary to synthesise new protein. The involvement of chain conformation of both DNA and RNA in this process is now widely appreciated but perhaps the most significant illustration of the relation between structure and function remains the Watson and Crick hypothesis of the double helical nature of DNA [2], the nucleic acid which serves as the repository for genetic information. It has been shown that when DNA duplicates one strand of the double helix goes to each daughter cell [32] where it serves as the template for the synthesis of the complementary strand. The process of unwinding of the double helix has been extensively studied in vivo [33] and it is from these studies that many of the techniques used to investigate the nature of ordered conformations of polysaccharides have been gleaned. Some of these techniques will be referred to alongside the analogous use on polysaccharides in later chapters.

D. Structure and Function of Some Polysaccharides

If the biological function of a polysaccharide is to be correlated with its structure, not only must its function be identified, but the structure of the molecule and its relation to other molecules must be determined. It has been stated that the biological function of a biopolymer is usually confined to its ordered state [34]. This observation is almost certainly true for both the biological function and the in vitro phenomena shown by the carbohydrate polymers studied in this thesis. Gum exudates (e.g. Acacia gum), on the other hand, are believed to exist entirely in disordered conformations, thus the biological function of these polymers probably depends on some property which arises from the random nature of the chain conformation.

The main polysaccharide function which has been clearly identified with structural order, both in the molecule itself and in the relation of the molecules to each other, is the ability to confer mechanical stability. This can be the very rigid mechanical stability which is characteristic of crystalline fibres joined in a sheath or net (e.g. cellulose) or the loosely reversible connections of a gel matrix (e.g. carrageenan in red seaweed). This function of imparting structure has been observed for polysaccharides in bacterial and yeast cell walls, in bacterial capsules and in various plant and animal tissues.

Carbohydrate polymers also serve as an energy store. Here again ordered structures may be important as they represent the most efficient method of packing polymer chains.

Many of the industrial uses of polysaccharides depend on properties which mimic their natural function[3] and thus many of these will also depend on ordered conformations of the chain. This is true, for example, in the use of cellulose fibres and in the formation of various gels.

Ordered conformations have been proposed for various carbohydrate polymers[35] on the basis of several physical techniques, especially X-ray diffraction.

Starch, an α-1,4-glucan, is composed of two components, amylose and amylopectin. Amylose, the unbranched component of this reserve polysaccharide, pre-empted both nucleic acids and proteins as the first biopolymer for which a helical structure was proposed.[36] There has been much controversy over the conformation adopted by amylose but it now appears as if some definite answers are emerging. It is recognised that in complexes with iodine and other ligands amylose coils round the molecule like a snake, giving what is known as the V form, or some expanded version of it [35]. The B form, which occurs in retrograded amylose is, as yet, a subject of great debate, however an attractive double helical structure has been proposed by French [37]. The conformation of amylose in solution will be discussed in Chapter III.

The reason why a reserve material like starch exists in two forms is unknown, but the situation parallels that in laminaran, the reserve polysaccharide of some seaweeds. Here again a branched and unbranched form exist. This polysaccharide and other β-1,3-glucans are discussed in Chapter III. Other reserve polysaccharides based on a mannan backbone are discussed in Chapter II.

Cellulose, the chief structural material of plant cell walls, is organised as partly crystalline fibrils embedded in an encrusting matrix of other polysaccharides.[38] The chains of this β-1,4-D-glucan are now generally accepted as adopting the "bent chain" conformation in these fibrils [39] (Fig. 0.5).

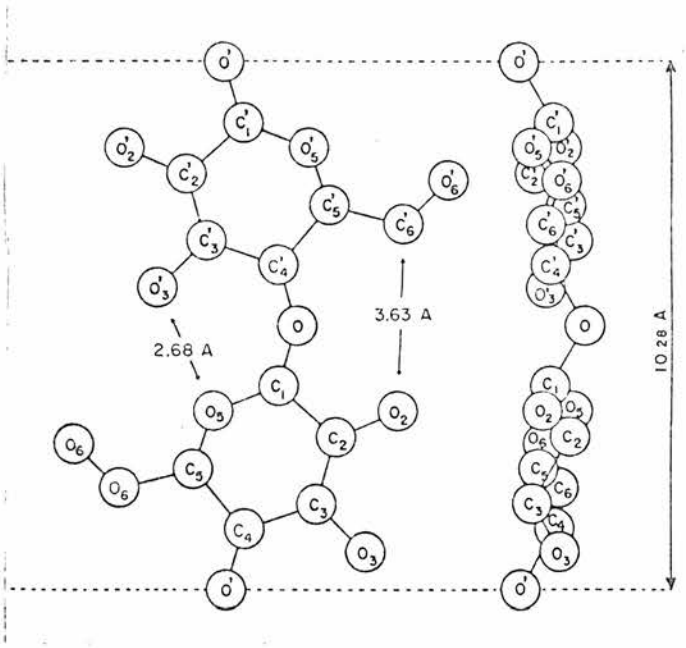


Fig. 0.5. The bent chain conformation of cellulose (from ref. 39)

Chitin, a β-1,4-linked polymer of 2-acetimido-2-deoxy-D-glucose, functions in fungal and bacterial cell walls, insect cuticle and the shells of crustaceans as the skeletal polysaccharide. It has been shown [39] that this polysaccharide adopts a conformation that is very similar to that of cellulose.

Certain other structural polysaccharides, which co-exist with cellulose as encrustants of the matrix have a backbone built up of β-1,4-D-xylose residues or a mixture of β-1,4-D-mannose and β-1,4-D-glucose residues. These xylans and glucomannans are often substituted by single unit carbohydrate side chains (usually L-arabinose and 4-O-methyl-D-glucuronic acid in the xylans and D-galactose in the glucomannans). In view of the fact that mannose is the C(2) epimer of glucose and that xylose is the pentopyranose analogue of glucose it is no surprise that these polymers have been shown to crystallise with ribbon-like conformations not far removed from that of cellulose itself [35].

Certain green seaweeds have been shown to utilise mannan or xylan, not cellulose, in their cell walls [40]. The chain conformation of the D-mannan resembles that of cellulose, which is expected as the residues are again linked β-1,4. The β-1,3-D-xylan, however, has been shown by X-ray diffraction studies to be arranged in triple helices. The details of this structure are discussed in Chapter III as it is analogous to that proposed for β-1,3-glucans.

Recent circular dichroism studies have shed some light on the conformation adopted by alginates, block co-polymers of 1,4-linked β-D-mannuronic and α-L-guluronic acid residues, when the polymer chains take part in junction formation during gelation [41]. The conformation that the chain segments adopt here is probably a reflection of the in vivo conformation of these segments of this structural seaweed polysaccharide. There is evidence [42] that pectin, a complex family of carbohydrate polymers based on a backbone of D-galacturonic acid residues linked α-1,4, behaves in a similar manner.

Mucopolysaccharides occur in various animal tissues (e.g. synovial fluid, connective tissue and the vitreous humour of the eye) and perform a variety of poorly understood functions such as the provision of support and flexibility, the absorption of shock at bone joints and perhaps the lubrication of these joints. One member of this structurally related family, hyaluronic acid, has been the subject of two recent X-ray studies on orientated films. Atkins et al [43] have shown that the molecule packs as an extended ribbon, whereas Rees and co-workers [44] have observed X-ray diffraction patterns which suggest the possibility of the molecules packing as double helices. Both of these structures may occur in vivo and perform a different function.

Plant mucilages, which are composed of a complex mixture of polysaccharides, have been shown to consist of particles with some internal crystallinity, and to denature in a manner analagous to globular proteins [45]. This ordered structure could be involved in the function of these tissues as a biological water store.

The extracellular polysaccharide from the plant pathogen Xanthamonas campestris has been shown to undergo a conformational transition when heated and cooled in aqueous solution [46]. This conformational change has an interesting effect on the viscosity of the material. It is found that the viscosity remains essentially constant with temperature, as the normal decrease in viscosity on heating is offset by the fact that a compact ordered conformation changes to an open fluctuating random coil. Although this industrially useful property is unlikely to be important in vivo it has been suggested

that this same ordered conformation is of importance in the pathological condition of cabbages produced by the bacterium. This condition results from mechanical blocking of water movement by the extracellular polysaccharide. Morris and Rees [46] have suggested that the congestion achieved by this highly viscous polysaccharide is aided by its interaction in an ordered conformation with the cellulose of the plant cell walls (see Chapter II).

In the carrageenans, the family of seaweed polysaccharides studied in the first two chapters of this thesis, a close relation has been found between the biological role, the industrial use, and the primary, secondary, tertiary and quaternary structure [3].

CHAPTER I

SOME ASPECTS OF THE DOUBLE HELIX TO COIL TRANSITION IN CARRAGEENAN

A. Introduction

The carrageenans constitute a family of sulphated galactans which occur as cell wall matrix components and in the intercellular regions of some species of Rhodophyceae (red seaweeds). It has been suggested that these polysaccharides may perform a variety of natural functions,[47] such as:

a) serving as a cushion of gel which protects the cells of the plant from the buffeting of waves and the rigours of tidal action;

b) providing a cation exchange barrier between the cell contents and the sea;

c) providing a store of sea water which helps prevent desiccation of the plant at low tide and rupture of the cells by osmotic stress in rain water.

Industrially these materials find use in food products, pharmaceuticals, cosmetics and many other areas.[48] Since many of the biological and industrial uses of these materials depend on their gel forming ability an intimate knowledge of the gelation process is imperative if we are to appreciate how these functions are achieved.

Primary Structure of the Carrageenans

Although early structural and physical studies of the carrageenans were performed on heterogeneous material, the order of magnitude of the molecular weight (several hundred thousand) found by Smith, Cook and Neal [49] in 1954 is none the less valid. Similar values have been obtained more recently.[50]

Over the last decade, however, the primary structures of the carrageenans have been elucidated, revealing that they are all linear polysaccharides which approximate to alternating copolymers of 3-linked β-D-galactopyranosyl and 4-linked α-D-galactopyranosyl residues. [52]

The carrageenan family has been divided into six members[51-53] (see below). While the hybrid nature of each type [54] is recognised this division is a valid one as all molecular species are based on one of these with slight variation in the degree of sulphation at a particular carbon atom or in the extent of anhydridisation of the 4-linked residue. The nature of the 3-linked residue provides a further useful division of the family. In kappa-, iota-, mu-, and nu-carrageenan this residue is generally β-D-galactose 4-sulphate but in lambda- and xi-carrageenan it is β-D-galactose-2-sulphate.

Lambda-Carrageenan

This non-gelling polysaccharide can be separated along with xi-, mu- and nu-carrageenan from iota- and kappa-carrageenan on account of its solubility in aqueous potassium chloride solution.

The structure has been shown to be essentially that depicted in Fig. 1.1.[51]

Xi-Carrageenan

The structure of this polysaccharide, which appears to replace lambda-carrageenan in some species, is similar to the latter but with full sulphation on C(2) of all residues and little or no sulphation on C(6) of the 4-linked residues.[53]

Kappa-Carrageenan

Kappa-carrageenan [Fig. 1.2 (a)] forms fairly rigid, thermally reversible gels in aqueous solution. Gelation, which occurs in the presence of K^+, Rb^+, Cs^+ and NH_4^+ ions but not Li^+ or Na^+ ions, shows marked hysteresis.[51] Some of the 3,6-anhydro-α-D-galactose residues are replaced by D-galactose 6-sulphate and both of these residues can be 2-sulphated. This "masking" of the repeating structure has a profound effect on the conformational properties of the polymer chain.

Iota-Carrageenan

Iota-carrageenan is almost structurally identical to kappa-carrageenan, the important difference being that in iota-carrageenan most of the 3,6-anhydro-α-D-galactose and D-galactose 6-sulphate residues are 2-sulphated [Fig. 1.2 (b)].[51] Iota-carrageenan gels are more elastic than kappa-carrageenan gels and show less tendency to syneresis.[55]

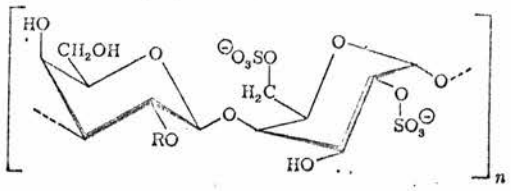


Fig. 1.1. Lambda-carrageenan R = H for some residues and SO_3^- for others.

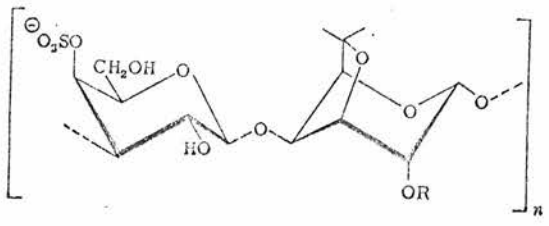


Fig. 1.2. (a) R = H Kappa-carrageenan

(b) R = SO_3^-. Iota-carrageenan

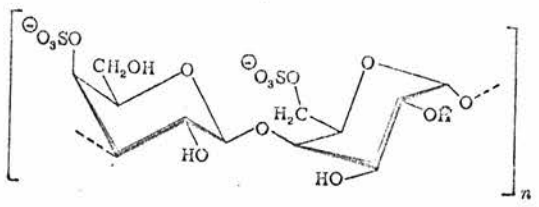


Fig. 1.3. (a) R = H Mu-carrageenan

(b) R = SO_3^- Nu-carrageenan

Mu-Carrageenan

Mu-carrageenan also has a very similar structure to kappa-carrageenan but the 4-linked residue is generally D-galactose 6-sulphate [Fig. 1.3 (a)] with only a small amount of 3,6-anhydro-α-D-galactose. This polymer is isolated with lambda-carrageenan in the potassium chloride soluble fraction of naturally occurring carrageenan and, in fact, the two are inseparable without chemical modification of mu-carrageenan.[51]

Nu-Carrageenan

Nu-carrageenan, which is yet to be isolated, is essentially mu-carrageenan with the 4-linked residue almost fully 2-sulphated [Fig. 1.3 (b)].[53] The structural features of the carrageenans have been illustrated by Stanley[56] (Fig. 1.4).

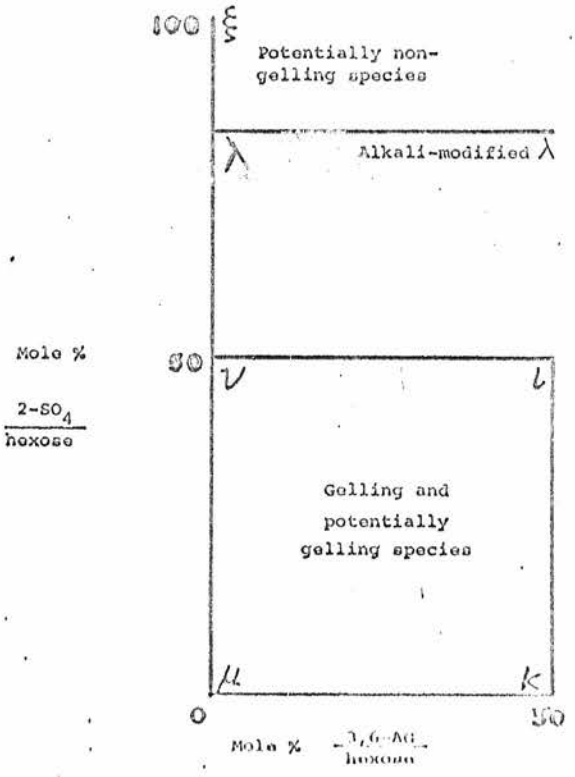


Fig. 1.4. Graphical illustration of the relation between the carrageenans in terms of the mole % of 2-sulphated residues and 3,6-anhydro residues.

Nu- and mu-carrageenan can be converted to iota- and kappa-carrageenan respectively by increasing the percentage of 3,6-anhydro 4-linked residues. The mu to kappa conversion can be achieved in the seaweed by an enzyme[57] and in the laboratory by the reaction described in section B of this chapter. Conversion of iota- to kappa-carrageenan does not appear to be possible.[58] Even in the laboratory the most recent desulphation techniques [59] cannot avoid some polymer degradation.

The Secondary and Tertiary Structure of Carrageenans

The discovery of the underlying regularity of carrageenan structure led to the suggestion that their solid state conformation could be determined by fibre diffraction methods.[60] The best data were obtained for iota-carrageenan where the α-D-galactose 6-sulphate and 2, 6-disulphate residues had been converted to the corresponding 3,6-anhydrides, but kappa-carrageenan treated in the same manner gave a photograph with remarkable similarities.[61] By use of computer model building methods, which start from the known covalent structure and search for the sterically possible chain conformations which will fit the symmetry and periodicity parameters obtained from the X-ray photographs, it was proposed that the chains occur in the fibre as double helices with three disaccharide residues per turn of each chain, this turn being 24.6 A^{o} (kappa-carrageenan) and 26.0 A^{o} (iota-carrageenan) along the helix axis. Strong support is given to this structure by physical models.[3] Both "ball and spoke" models and "Corey-Pauling-Kolton" space filling models show:

a) a lack of steric compression;

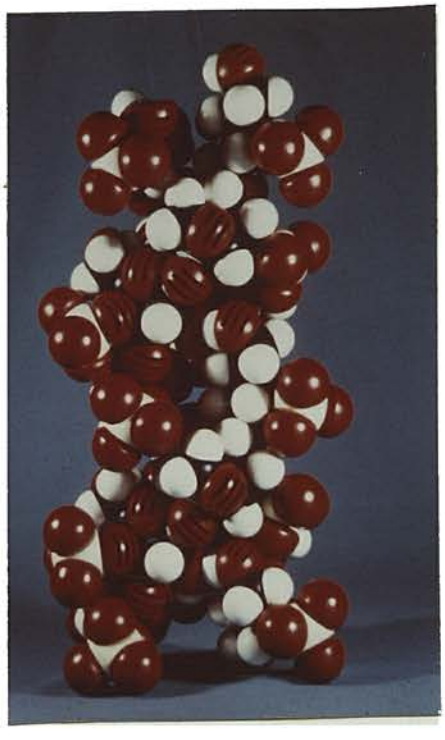
b) the possibility of an interchain hydrogen bond between the C(6) hydroxyl of each β-D-galactose 4-sulphate and the C(2) hydroxyl of an equivalent residue on the second chain;

c) that the hydrophobic surfaces are tucked into the inside and the bulky hydrophilic sulphate groups are on the outside (see Fig. 1.5).

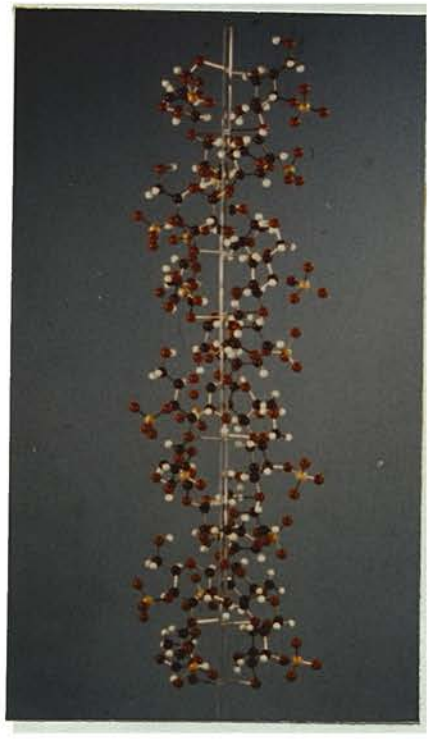
The presence of an interchain hydrogen bond inside the helix and at right angles to the helix axis has been verified by deuteration and infrared dichroism.[62]

The reason why poor X-ray photographs are obtained when α-D-galactose 6-sulphate and 2,6-disulphate residues are present to an appreciable extent has been shown to be that these residues cannot be incorporated into the double helix[63] as they adopt the Reeves C1, not the 1C, chair. This causes the chain to change direction and a kinked helix would be obtained but conversion to the anhydride (1C chair) allows formation of uninterrupted helices which "crystallise" more easily.

The discovery of the double helix, coupled with the effect of changes of primary structure on gelation,[64] led to the proposal that the tie points of the gel network are provided by double helix junctions as shown in Fig. 1.6.



(a)



(b)

Fig. 1.5. The iota-carrageenan double helix in (a) Corey-Pauling-Koltun and (b) "ball and spoke" models.

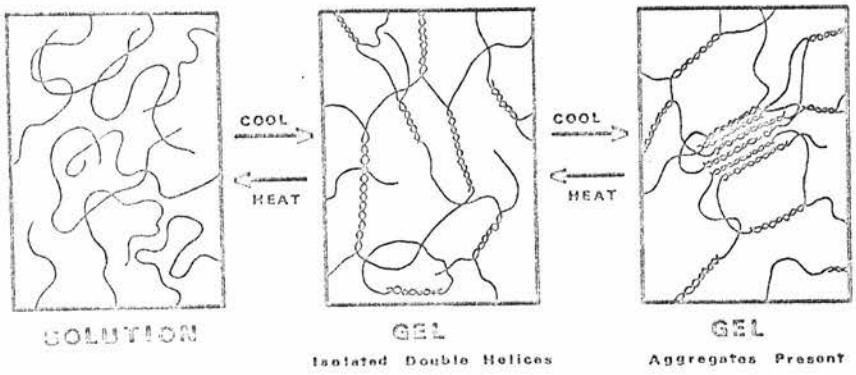


Fig. 1.6. Mechanism of gelation by carrageenans proposed by Rees et al[63].

As carrageenan gels liquify when heated and set when cooled it was proposed that a double helix to coil transition is taking place. Experiments which follow the change of some parameter which depends on chain shape tended to confirm this view.[62] Optical rotation (O.R.) measurements on kappa-carrageenan, as with other biopolymers undergoing a helix to coil transition,[33] show a sharp change in the region of the gel point on heating and cooling.[63] Unfortunately these O.R. changes are complicated by turbidity, gel formation, cell strain and perhaps stress birefringence. Further evidence of the importance of a helix to coil transition in gelation was obtained after chemical degradation at the "kinking"residues.

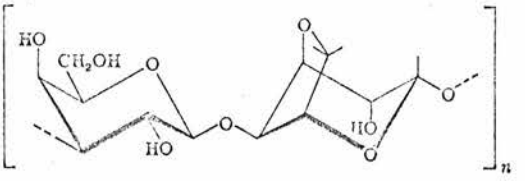
The ideal sugar units of iota carrageenan are unaffected by Smith degradation but α-D-galactose 6-sulphate contains adjacent hydroxyl groups. Thus it was possible to segment the chains and after conversion of the "kinking" residues short, idealised chains were obtained. These chain segments gave a product which did not gel but still displayed sharp changes in O.R. on heating and cooling.[65] This O.R. shift was attributed to a change in the chain conformation which occurs by rotation about the bonds between the sugar residues and not by changes in the residues themselves. It has been shown that the observed shift agrees with that predicted by an empirical O.R. treatment of such changes for the coil to double helix transition.[66] This empirical treatment [67] is discussed in Chapter III where it is applied to another family of polysaccharides, the β-1,3-glucans.

It has been suggested that the chains form a gel network (i.e. unite in a double helix with more than one partner) as "kinking" residues prevent complete zipping up of a pair of molecules, and force the remainder of the molecule to link up with another partner.[63] This behaviour is reminiscent of the role of proline and hydroxyproline residues in terminating α-helical sections of globular proteins.

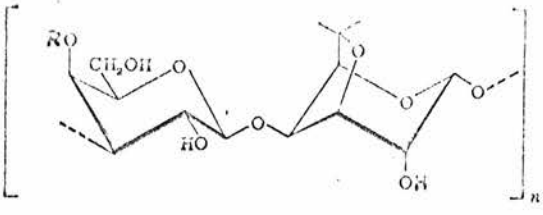
Recently another piece of supporting evidence for the existence of a double helix to coil transition in solution has come from molecular weight studies of Smith degraded iota-carrageenan.[68] It has been shown, by light scattering and membrane osmometry measurements, that the molecular weight of this carrageenan doubles when cooled through the O.R. transition.

The Structure of Kappa-Furcellaran and Agarose

The idealised structures of these gel forming polysaccharides (Fig. 1.7) are shown here, since many observations on these materials are used in the discussion of carrageenan behaviour. Both, but especially kappa-furcellaran, are closely related to the carrageenans and perform the same function in seaweeds. It seems probable that both adopt a double helical structure capable of further aggregation [51] and this will be assumed in later discussion.



(a)



(b)

Fig. 1.7. (a) Agarose

(b) Kappa-furcellaran. R = H for about 50% of the residues SO_3^- for the others.

B. EXPERIMENTAL

Materials

These are described in the "General Methods" section.

Preparation of Smith-Degraded, Alkali Modified Kappa-Carrageenan.

Smith degradation is based on the work of Goldstein, Hay, Lewis and Smith [69] and the alkali modification on that of Rees.[70] The reaction sequence is illustrated in Fig. 1.8. Kappa-carrageenan (25 g; REX 5401) was dissolved in water (4 litres) by boiling for several minutes, and then mixed at room temperature with sodium metaperiodate (53.5g.) in water (1 litre). After allowing the oxidation to proceed in the dark at room temperature for 65 hours, an excess of ethylene glycol was added. Sodium borohydride (75g.) was dissolved in the solution which was left at 2°C for 72 hours, and then made 1N with respect to sodium hydroxide before heating for 5 hours at 80°C, with further addition of borohydride (25g.). This last step is the alkali modification which converts 6-sulphate to 3,6-anhydro residues. The mixture was cooled, neutralised with hydrochloric acid and then excess acid was added to pH 1. After 24 hours at room temperature for the selective hydrolysis step, potassium hydroxide solution was added to neutrality before dialysis against running tap water for 6 days, in the presence of thymol as preservative. The solution was dialysed against daily changes of distilled water for 6 days, then passed through a column of Amberlite I.R. 120 ion exchange resin (K^+ form) and evaporated under reduced pressure to 6 litres.

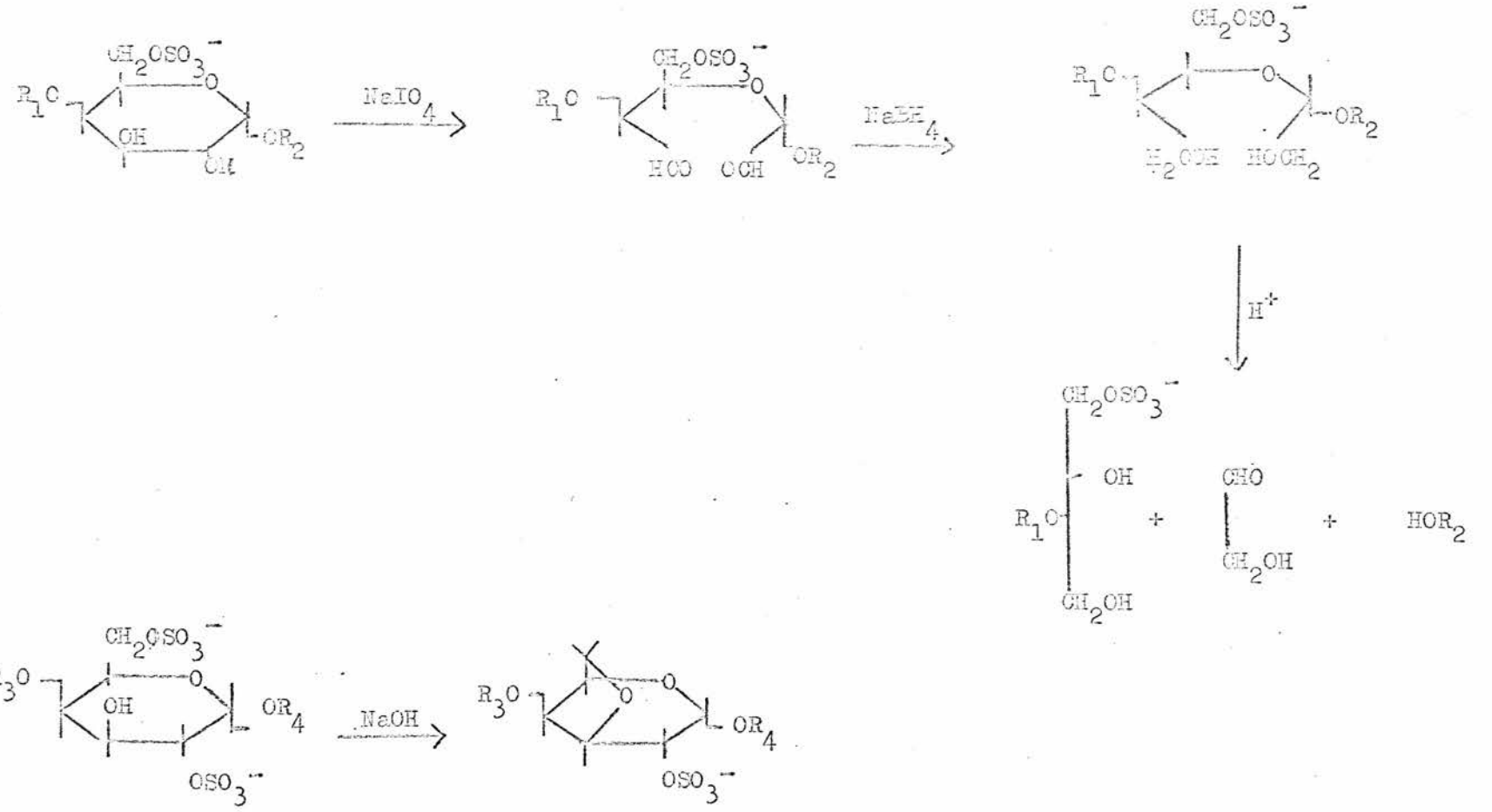


Fig. 1.8. Fate of "kinking residues " (D-galactose-6-sulphate and D-galactose-2,6-disulphate) during Smith-degradation and alkali modification.

Fractionation of the Smith-Degraded, Alkali Modified Kappa-Carrageenan.

To the 6 litres of solution obtained from the above degradation, potassium chloride solution (2M; 240 ml.) was added followed by isopropanol (480 ml.). After warming to 70°C, the solution was cooled slowly and filtered. The precipitate, which represented 32.5% of the total carbohydrate content (phenol-sulphuric assay [71] described in "General Methods"), was collected on the centrifuge, dissolved in water and dialysed against running tap water for 7 days and then against daily changes of distilled water for a further 7 days. The solution was passed through an ion exchange resin (I.R. 120, K^+ form) then freeze dried.

Addition of isopropanol (420 ml.) to the supernatant solution precipitated a further 30% of the polysaccharide. This product was worked up in the same way as the previous fraction and is the middle fraction used in the experiments described below. The product left in the supernatant from this material was also converted to the K^+-form and isolated.

Preparation of Smith-Degraded Agarose

Agarose (2g; REX 5468) was dissolved by autoclaving in water (300 ml.) and then mixed at 40°C with sodium metaperiodate (5.35g.) which had been predissolved in water (200 ml.). Oxidation was allowed to proceed for 75 hours at this temperature before addition of excess of ethylene glycol to halt the reaction, followed by potassium borohydride (6g. in 2.5 litres of water). After two days the solution was dialysed against running tap water for 5 days and adjusted to 0.1 N

with respect to hydrochloric acid for mild hydrolysis which was allowed to proceed at room temperature for 16 hours. The solution was neutralised with potassium hydroxide, then dialysed again for 7 days against running tap water before evaporation under reduced pressure and freeze drying.

Determination of Gel Setting and Melting Points.

This was carried out as described in the "General Methods" section. The results are shown in Table 1.1 and in the corresponding O.R. versus temperature plots for the same samples.

Measurement of Optical Rotation versus Change of Temperature for Kappa-Carrageenan Solutions.

These measurements were carried out on three samples of kappa-carrageenan, one from Chondrus crispus, which contained some 2-sulphate on the 4-linked residue, another from Eucheuma cottonii, which contained no 2-sulphate, the third sample being the middle fraction of the Smith-degraded alkali modified preparation of the Chondrus crispus sample. All three were in the potassium salt form. The preparation and clarification of the solutions and the measurement of optical rotation against temperature are described in the "General Methods" section. O.R. measurements were not corrected for changes in refractive index or solvent density with temperature as this was found to make no difference to the overall picture. 0.1 dm. cells were used in all cases. The results are shown graphically at the end of the chapter (graphs 1.1, 1.2 and 1.3).

Measurement of Optical Rotation versus Change of Temperature for Iota- and Lambda-Carrageenan Solutions.

This was carried out using the same procedure as for the kappa-carrageenan samples. The results are shown on graphs 1.4 and 1.5.

Optical Rotation versus Temperature for Carrageenan in Potassium Chloride Solution.

The procedure for kappa-carrageenan in water was repeated for 0.3%, 0.6% and 1.0% (weight/volume) (w/v) potassium chloride solution. See graph 1.6.

Investigation of the Time Dependence of Points on the Kappa-Carrageenan Hysteresis Loop.

A hot solution to be studied was millipore filtered into two hot cells for which cell blanks had been determined. One of these cells was heated to 70°C for two hours and the other was held at room temperature overnight. Both cells were then quickly connected to a bath which circulated water at a fixed temperature. Optical rotation measurements were then recorded against time. See graphs 1.7 and 1.8.

"Scanning Curves"

The 3% w/v kappa-carrageenan system (graph 1.1) was cooled from 70°C to a point on the hysteresis loop. The change in O.R. on reheating

to 70°C was then plotted giving a descending scanning curve.
The results are shown in graph 1.9. Ascending scanning curves were plotted by heating the system from 20°C to a point on the loop and following the change of O.R. as the system was cooled to 20°C.
The results are shown in graph 1.10.

C. Results and Discussion

1. The General Shape of the Optical Rotation Transition.

Early O.R. investigations of the temperature induced helix to coil transition of kappa-carrageenan produced curves of the shape shown in Fig. 1.9. [63]

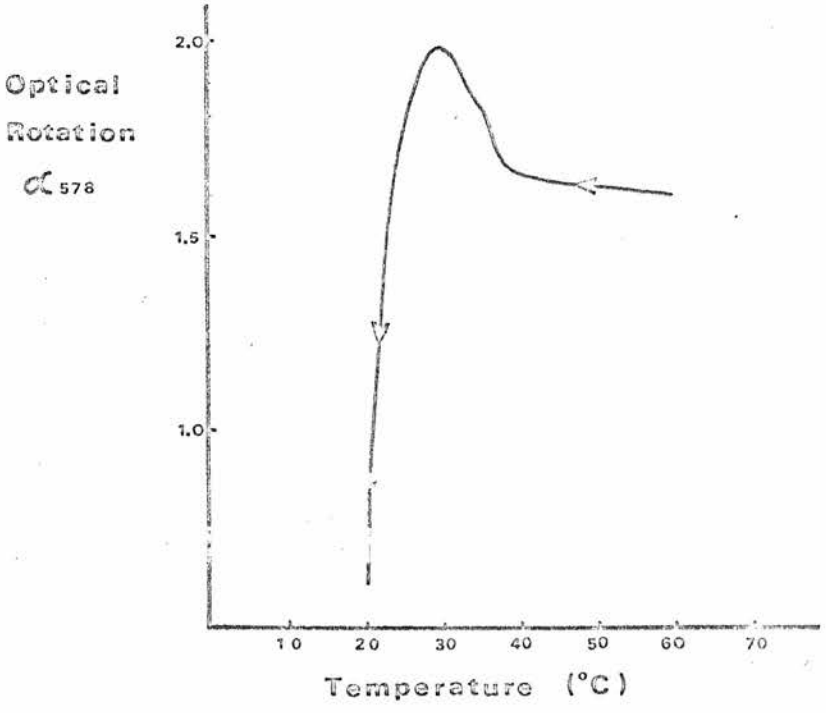


Fig. 1.9. Optical rotation changes on cooling a hot solution of kappa-carrageenan (from ref. 63).

It now appears that the turnover and subsequent reduction of the optical rotation as the temperature is lowered is caused by the lack of clarity of the solutions and is not due to a negative contribution from some secondary process of gel formation such as aggregation or stretching of the chains due to tightening of the gel. Lack of clarity can produce at least four types of effect on the O.R. measurements.

a) If the photocell of the polarimeter is receiving a weak signal it does not respond correctly.

b) If there are particles in the solution it is possible that the light beam may not be intense enough to penetrate to the molecules in the centre of the particles.

c) Such particles could cause a shadow to form behind them (see Fig. 1.10). The molecules in this shadow will not be "seen" by the beam.

d) A fourth artifact can arise from the effect of reflection from a particle on the polarisation of left and right circularly polarised light. [72]

These effects have been recognised and to some extent corrected for in circular dichroism measurements on suspensions of biological membranes. [73]

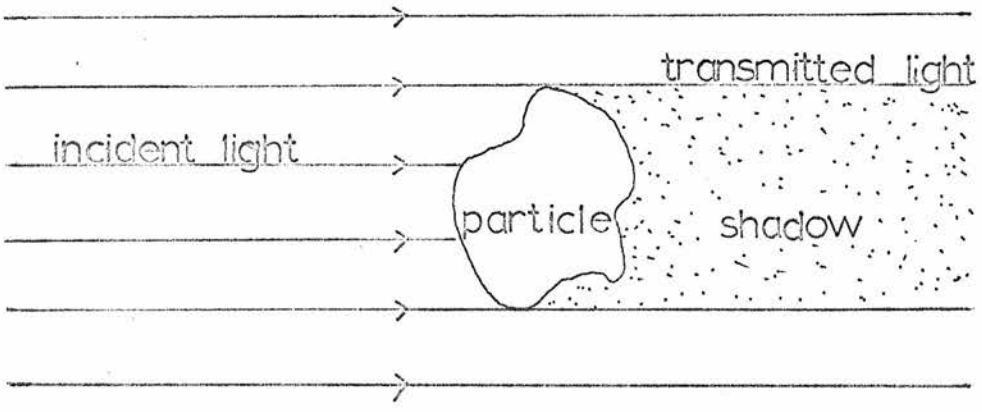


Fig. 1.10. Effect of opaque particles on O.R. measurements. Material both in the particle and in the shadow may not be "seen" by the beam.

Such artifacts can be largely overcome by millipore filtering to remove insoluble material. Two simple manoeuvres may also be used to recognise the existence of such artifacts. The first depends on the fact that the O.R. measurements can be taken at several wavelengths. Since the intensity of the light beam is much higher for some wavelengths than others it is found that the measurements using the lower intensity beam are found to deviate from those of the higher because they cease to penetrate the particles sooner. If measurements at both wavelengths show parallel changes with temperature it can be assumed that effects of type (a), (b) and (c) are unlikely to be present. The other method involves a variation of the cell pathlength, for example by use of 1 dm. and 0.1 dm. cells. If the results are proportional to pathlength it seems likely that there is no interference of the type

produced by (a). These methods do not completely rule out artifacts but they do reduce their likelihood to a minimum.

2. Smith-Degraded Kappa-Carrageenan and Agarose.

The three fractions of Smith-degraded (segmented) kappa-carrageenan were prepared as described. The middle fraction was used in all experiments described below.

This material dissolved easily and showed no tendency to come out of solution on cooling although it still underwent an O.R. transition when heated and cooled. Even concentrated solutions 4% w/v were very mobile and showed no tendency to gel. However very concentrated solutions tended to aggregate and gel when potassium chloride was added.

The low viscosity of even the 4% w/v solution at low temperature could be due to the shortness of the segments or to an effect similar to that proposed for the extracellular polysaccharide from Xanthamonas campestris.[46] Normally the viscosity of a polymer solution increases on cooling, but if an ordered structure is formed, the reduction in the hydrodynamic volume of the polymer chains will tend to decrease the viscosity and could dominate the effect.

Reduced hysteresis is retained over part of the transition (graph 1.3). This behaviour is discussed later in this chapter. Apart from hysteresis the properties of this material are very similar to those of segmented iota-carrageenan.[65]

Comparison of the transition with that of undegraded kappa-carrageenan (graph 1.1) shows it to be sharper and the total change in O.R. to be larger. The greater magnitude of the transition for a given concentration must reflect the regions of the undegraded material which are prevented from helix formation by too high a density of kinking residues, or because of the release of physical restraints encountered in a gel. The presence of regions rich in kinks has been inferred from molecular weight studies on segmented iota-carrageenan.[68]

Removal of restraints imposed by gel formation could also explain the increased sharpness of the latter part of the transition for more concentrated solutions. In solutions of lower concentration the increased sharpness could be a result of the molecular weight fractionation. There is also a possibility that idealisation of the chemical structure by alkali modification could reduce the breadth of the transition by decreasing the range of transition points shown by species of different primary structure.

The retention of the O.R. transition even though gel formation is abolished dispels doubts about the O.R. changes arising from cell strain, stress birefringence and other bulk effects.

Segmented agarose also dissolved when heated in water, but it precipitated from solution on cooling. Although this material was soluble in 3M guanidine hydrochloride at low temperature, no optical rotation transition was observed in this solvent. No intermediate concentration of guanidine hydrochloride could be found in which the O.R. transition was observed without simultaneous aggregation.

The preparation and behaviour of carrageenan segments parallels similar investigations on cyanogen bromide peptides of collagen. [74]

3. Hysteresis

(a) Historical

The term hysteresis was introduced by Ewing [75] to describe the phenomenon in which an effect lags behind the cause. The classic example of hysteresis occurs in magnetisation of ferromagnetics but it has been observed in many other systems. [76] In fact Everett [77] points out its applicability in such areas as population movement, political science and memory processes. The relation between hysteresis and memory has been further developed by Katchalsky and Oplatka [78] with reference to the hysteresis of the pH titration curve of r-RNA.

A general approach to hysteresis has been developed by Everett and Whitton. [76] They point out that for a system in equilibrium any change from a state A to a state B will proceed reversibly. Systems in which changes do not proceed reversibly, regardless of how slowly they are carried out, are said to exhibit hysteresis. Everett and Whitton distinguished time dependent and time independent hysteresis. In time independent hysteresis all points on both paths A to B and B to A are stationary. When this criterion is not fulfilled, the process is said to exhibit supersaturation or metastability. The time dependent phenomenon is typified by the volume - temperature relationship for the melting and freezing of a pure substance (Fig. 1.11). The path ABDE is stable as is the first

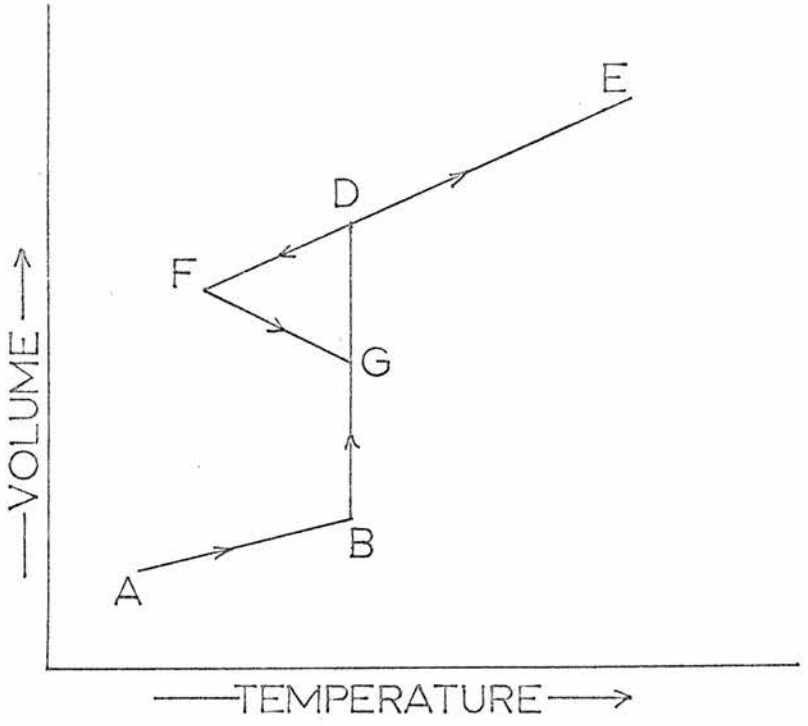


Fig. 1.11. Volume - temperature relationship for the melting and freezing of a pure substance.

part of the reverse path ED. The portion DF (supercooling) is a metastable process and at some point F solidification commences. No stable points can be observed on the path FG but points on GBA are stable.

(b) Everett's Domain Model of Hysteresis

Although Everett and Whitton distinguished the terms hysteresis and metastability they recognised that the two were closely related. Using a mechanical model (Fig. 1.12) they postulated that

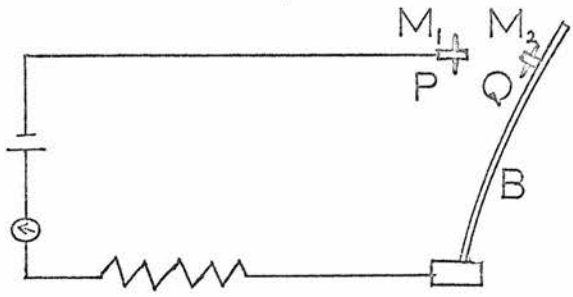


Fig. 1.12. Everett's mechanical model.

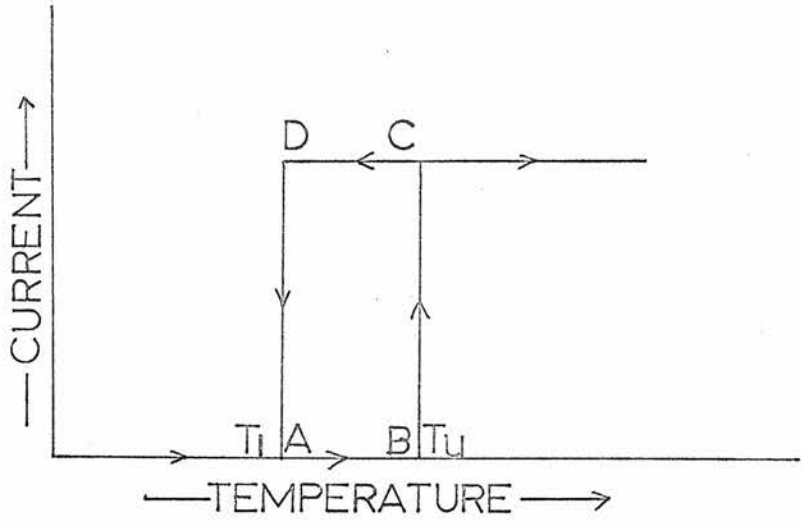


Fig. 1.13. Graphical representation of the behaviour of this model.

hysteresis is attributable to the existence of a large number of independent domains all or some of which exhibit metastability.

The model comprises two contacts in an electrical circuit, P and Q, attached to two magnets M_1 and M_2. One of the magnets is fixed while the other is attached to a bimetallic strip B. As the temperature is increased Q bends towards P and at a temperature T_u, when the magnets are close enough, they facilitate the closing of the circuit. On cooling the circuit does not open at T_u but the temperature must be lowered to T_l where the stress in B overcomes the magnetic attraction and the switch opens. This behaviour is represented in Fig. 1.13 where the current flowing is plotted against the temperature T. Thus this system shows metastability as points on BC and DA are not stable. The energy barriers imposed by the magnetic attraction and the rigidity of the bimetallic strip force the system to follow a non-equilibrium path. The joint operation of a large number of such switches with a distribution of opening and closing temperatures would give a smooth continuous and reproducible curve. Thus the whole system displays hysteresis despite the fact that individual switches exhibit metastability. Three important conditions must be satisfied:

(i) the switches must not be identical;

(ii) the switches must be independent (the closing of one must not cause co-operative closing of the others);

(iii) the operation of one switch must not be large enough to be seen on its own.

Therefore the switches, or more generally the domains, must be small, independent and non-identical.

(c) Hysteresis of Molecular Systems

Hysteresis of molecular systems can be represented on a free energy surface [63,76] (Fig. 1.14). The surface has two valleys which overlap in the direction of the independent variable and each valley turns into its neighbour at a point of inflection. If these valleys correspond to the states A and B, then hysteresis arises due to the persistence of a metastable state when changing from A to B and/or from B to A. This barrier, which is the rigidity of the bimetallic strip in one direction of the mechanical model and magnetic attraction in the other direction, represents the energy of the transition state. When hysteresis occurs this transition state is unattainable on a practical time scale. In truly reversible chemical and biochemical reactions the attainment of the transition state dictates the speed of reaction.

Two additional factors must be considered for molecular hysteresis:

(i) molecules do not all have the same energy as each other and although the average energy of the system may be insufficient, individual molecules may have sufficient energy to surmount the barrier and so cause the metastable state gradually to disappear;

(ii) the domains may not be entirely independent; change of one domain may help to bring about changes in its neighbours.

Fig. 1.14. Schematic free-energy surface for the melting and setting of carrageenan gels. The system follows the path shown by the arrows. Axes are labelled G (relative Gibbs free-energy), S (entropy) and T (temperature). It is not suggested that the absolute free energy of any species decreases with temperature nor that the entropy of the transition state is constant with changing temperature.

Fig. 1.15. Modified free energy surface (see text.)

(d) Hysteresis of Carrageenan Systems

Polysaccharide gels of agarose, furcellaran and kappa-carrageenan display hysteresis in their melting and setting behaviour (Table 1.1). The O.R. changes which accompany the sol to gel transition of aqueous kappa-carrageenan also exhibits hysteresis (graphs 1.1 and 1.2). Hysteresis could arise from metastability of the system on gel melting, gel setting or both. Gel melting is very like the melting of true crystals and is thus expected to be an equilibrium process. [51] For this reason and others, which are mentioned under "Time Dependence of Hysteresis", gel melting and hence the melting of helices are considered to be equilibrium processes. This simplifies the energy surface (Fig. 1.15) as all points in one valley are always below the equivalent point in the other until the transition temperature is reached, i.e. the helix never exists as a metastable state.

Gel setting involves helix formation and subsequent aggregation of these helices. Both processes could theoretically give rise to hysteresis. Nucleation of a double helix or an aggregate could present a barrier to the formation of the species with lowest free energy. This barrier would be lowered on decreasing the temperature because such negative coefficients of reaction rate are characteristic of processes initiated by nucleation although they are not observed for usual chemical reactions. Rate studies on the formation of the DNA double helix [79] and the collagen triple helix [80] show the necessity of the formation of unstable nuclei (the first few turns of the helix) before the molecules eventually zip up. These studies also show that the maximum reaction rate occurs well below T_m, the centre of the coil to helix transition.

Although helix nucleation was originally believed to be the origin of carrageenan hysteresis [63] several observations now suggest that aggregation of the helices themselves is more likely to be the important factor.

(i) In the series of related polysaccharides agarose, kappa-furcellaran, kappa-carrageenan and iota-carrageenan the tendency to form turbid gels (a characteristic of aggregation of the polymer network) parallels the magnitude of the hysteresis observed on setting and melting these gels. [55] Iota-carrageenan in fact shows no evidence of aggregation, and hysteresis if present is undetectable (see graph 1.4).

(ii) The turbidity of the gel of a particular kappa-carrageenan is found to depend on the degree of 2-sulphation of the anhydride residue and as shown by graphs 1.1 and 1.2 the magnitude of the hysteresis loop follows the same trend. Similar behaviour is shown in the agarose series, using samples with varying O-methyl substitution.[81]

(iii) As judged by the turbidity of the system, the aggregated nature of kappa-carrageenan gels can be increased by increasing the concentration of the polymer or by adding potassium chloride to the system. It is immediately obvious from graph 1.6 and Table 1.1 that addition of KCl also increases hysteresis. Polymer concentration has a marked effect on the tendency of a system to show hysteresis (graphs 1.1 and 1.2) and lowering the concentration of one kappa-carrageenan below 2% (w/v) was found to reduce hysteresis to an extent where it is almost undetectable (graph 1.1).

(iv) Nucleation of one domain by another [see section (h)] has been shown in carrageenan systems. It is easy to envisage that double helices could overcome nucleation barriers by binding to pre-existing aggregates but no process can be suggested to account for the existence of one double helix aiding formation of another except through such aggregation.

(v) As stated earlier, Smith-degraded kappa-carrageenan shows reduced hysteresis in its O.R. behaviour. Here again the extreme clarity of these solutions indicates an empirical link between aggregation and hysteresis.

As in the case of DNA [79] and collagen [80] the barrier presented by helix nucleation is probably insufficient to prolong the existence of a metastable state.

(e) Possible Nature of the Domains of Kappa-Carrageenan Systems

Several possibilities may be suggested.

(i) They could be helices which are physically separate in the gel or solution.

(ii) They could be stable aggregates of a certain size.

(iii) They could be groups of molecules with the same transition temperature. Different groups of molecules with different transition temperatures could arise from variations in primary structure.

(f) Free Energy Surface

If we now follow a carrageenan solution around a heating and cooling cycle we see how hysteresis arises in terms of the energy surface (Fig. 1.15). As on the model of Rees, Steele and Williamson [63] the change between two states of the polysaccharide chains are considered, the random coil conformation (valley BC) and aggregated helices (valley AX). Other states exist on the barrier and will be discussed later. At high temperature the large entropy of the coil makes this the favoured state.

$$\Delta G = \Delta H - T\Delta S$$

The importance of this $T\Delta S$ term diminishes as the temperature is lowered causing the free energy of the aggregated helix to decrease faster than the free energy of the coil. At a point Y the free energy of both states is the same and if equilibrium was attainable the molecules would distribute between the two states. The transition state is however unattainable at this temperature and further cooling to a point where the barrier is surmountable is necessary before the aggregated helix is formed at C. Reheating takes the system along the equilibrium path AXYB from the aggregated helix to the coil.

Thus far the nature of the barrier between the random coil and the aggregated helix has not been discussed. The system must pass through:

(i) the helix nucleus;

(ii) the double helix;

(iii) the aggregate nucleus.

(i) and (iii) are unstable states by definition and must therefore correspond to energy maxima. It has been argued in the preceding section that the energy maximum for the aggregate nucleus is likely to represent the highest point overall. This then leads to two related possibilities. Firstly, the double helix could exist (over a certain temperature range) as a separate entity before aggregation. Secondly, the double helix could be an unstable state which is incapable of prolonged independent existence. These two possibilities can be shown in terms of the S versus G (i.e. entropy versus free energy) profile which is an isothermal cross-section of the surface shown in Fig. 1.15. At some point between AC and XY the two alternative mechanisms are characterised by profiles such as in Fig. 1.16; in (a), if the helix is capable of independent existence before aggregation, and in (b), if it is not. Thus it may be that, as in Fig. 1.16a, we should consider a three valley surface with two of these valleys (the isolated helix and the coil) in constant equilibrium.

Comparison of light scattering and O.R. measurements on cooling a solution of kappa-carrageenan did suggest earlier that double helix formation occurs several degrees before aggregation.[63] Such isolated helices would be expected from the mechanism shown in Fig. 1.16a, or if some molecules of kappa-carrageenan are iota-like in character and form double helices which are incapable of further stabilisation by aggregation. The S versus G profile of the latter situation is shown in Fig. 1.16c. These measurements also suggested that double helices remained on heating after the disappearance of aggregates.[63] The existence of these isolated helices at a temperature where they do not exist on cooling can only be explained by hysteresis arising from the

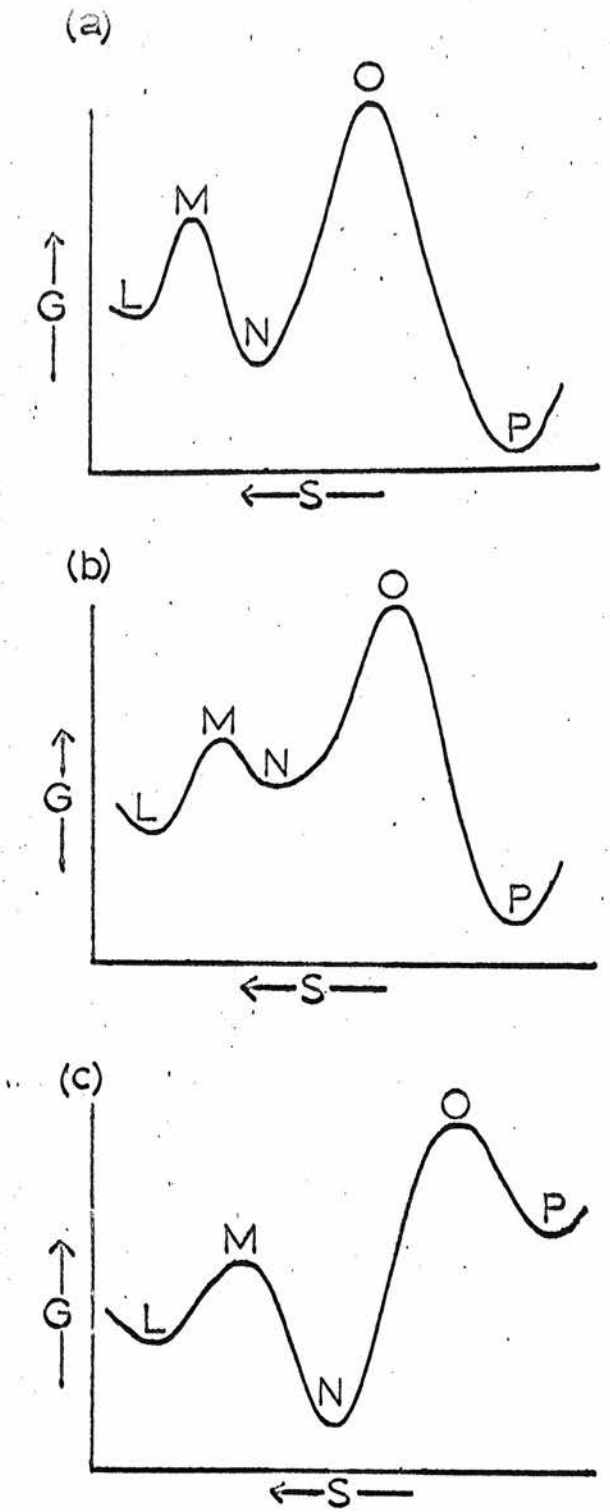


Fig. 1.16. Possible entropy (S) versus free energy (G) profiles between the random coil, the double helix and the aggregated double helix. L, N and P are the free energies of the random coil, the double helix and the aggregated double helix respectively. M and O represent the helix and aggregate nuclei. See text.

difficulty of helix nucleation. However a more detailed examination should be made of the correlation of O.R. and light scattering because the distorting influence of artifacts on the O.R. transition was not fully understood in the earlier work, [63] and because all the new evidence that is given in this thesis indicates that the origin of hysteresis is in the nucleation of aggregates.

I shall now attempt to explain various properties of the carrageenan system in terms of the above mechanism.

(g) Time Dependence of Carrageenan Hysteresis

Molecules have a distribution of thermal energies (Maxwell-Boltzmann distribution) as depicted in Fig. 1.17.

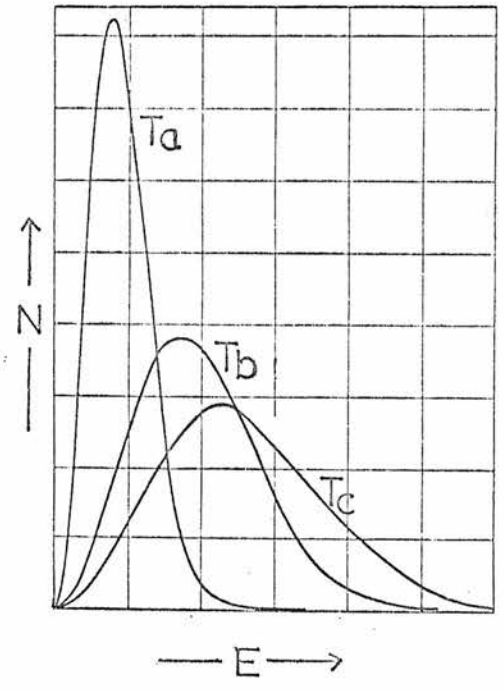


Fig. 1.17. Variation of the Maxwell-Boltzmann distribution of thermal energies with temperature. The number of entities (N) with a given thermal energy (E) is shown for three temperatures where $T_c > T_b > T_a$

This means that while in the metastable state (points on YC Fig. 1.15) some molecules may attain sufficient energy to surmount the barrier O (Fig. 1.16a,b) without change in the average energy of the system. This is demonstrated rather neatly for kappa-carrageenan systems. In concentrated gels (4% w/v) all points on the hysteresis loop are stationary for at least 14 days. In this system gelation damps the effect expected from the Maxwell-Boltzmann distribution by tying down the molecules. In a 3% gel, however, points on the cooling curve are not so stable and for example, hysteresis at 40°C (graph 1.7) has vanished after approximately 2 weeks. This meeting of the two boundaries of the hysteresis loop by movement of the cooling curve only, provides compelling evidence for the equilibrium nature of the melting process. The fact that points on all the heating curves remain stationary (graphs 1.7, 1.8) supports this view.

The hysteresis observed for segmented kappa-carrageenan is small at all concentrations and vanishes after approximately 3 days (graph 1.8). This system in which gel constriction cannot exist, shows little concentration variation of time dependence and, in fact, behaves like a weak solution of undegraded material.

(h) Nucleation of One Domain by Another

Several observations have led to the conclusion that kappa-carrageenan and agarose systems do not have fully independent domains.

(i) It has been shown by differential scanning calorimetry that solutions of kappa-carrageenan which were prepared by heating dispersions of the polysaccharide at 90°C undergo the coil to

double helix transition at higher temperature than solutions prepared by pressure cooking.[82] Therefore the former solutions probably contain material that can act as a template for helix formation.

(ii) Experiments on agarose show that similar seeding of gelation is possible.[83]

(iii) Hysteresis of agarose systems is time dependent only when some of the transformed material already exists in solution i.e. instability of points on the cooling curve is observed in the region BC and not on AB[81] (Fig. 1.18).

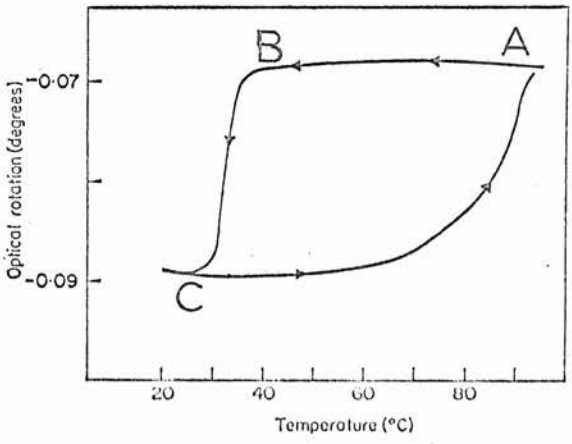


Fig. 1.18. Hysteresis in the optical rotation behaviour when heating and cooling an agarose solution (from ref. 116).

These observations must imply that the species on top of the activation barrier can be stabilised by interaction with existing helix aggregates, presumably by binding.

(j) "Scanning Curves"

Everett and Whitton [76] point out that hysteresis phenomena are characterised by scanning curves which traverse the hysteresis loop from the heating to the cooling branch and vice versa. Such scanning curves are observed for carrageenan systems (graphs 1.9 and 1.10). The descending curves converge on the lower intersection point of the main loop and the ascending scanning curves on the upper intersection point of the main loop. According to the second of Everett and Smith's general theorems on hysteresis [77] such behaviour is indicative of a wide distribution of domain properties.

It would thus appear that these systems exhibit some properties which characterise hysteresis and some which characterise supercooling.

(k) Comparison with Conformational Hysteresis of other Biopolymers

(i) Poly (α-L-Glutamic Acid) [84]

O.R. versus temperature curves for poly (α-L-glutamic acid) have been shown to display hysteresis, which has also been studied by light scattering, sedimentation, viscosity and chromatography. The hysteresis of this system is based on the difficulty of nucleating the aggregation of single α-helices of poly (α-L-glutamic acid). There is

thus a striking similarity with the carrageenan system - but also one important difference; aggregation of poly (α-L-glutamic acid) is thought to provide a real change in O.R., whereas such changes appear only as artifacts in the kappa-carrageenan case.

(ii) Sol - Gel Transformation of Methylcellulose in Water [85]

Turbidity measurements on the sol to gel transformation of aqueous methylcellulose solutions have been shown to exhibit marked hysteresis. Methylcellulose gels form by aggregation, on heating, of previously solvated chains. The aggregates form because there is an entropy increase with break up of water structure around individual chains. As in the other systems there is the possibility of nucleation of aggregation causing hysteresis.

(iii) Ribonucleic Acid

Proton Relaxation in the Interaction of t RNA with Manganese Ions [86]

Hysteresis of proton relaxation has been observed in the interaction of t RNA with Mn^{2+} ions during thermal denaturation and renaturation. Here again aggregate formation has been put forward as the origin of the hysteresis and suppression of opalescence in this system is accompanied by suppression of hysteresis.

pH - Titration of r RNA [87]

Cox and Katchalsky have postulated an entirely different mechanism for the hysteresis in the pH-titration of several r RNAs.

The secondary structure of r RNA is known to consist of intramolecular double helical sections linked by single stranded regions, but in this case no aggregation of this secondary structure is involved. Hysteresis is attributed to a small number of regions of adenine and cytosine residues, both of which are known to form stable complexes with themselves in acidic solutions. Thus at low pH when double helical regions melt these complexes form and on raising the pH do not break up at the same pH as they form. Hysteresis could thus be due to the difficulty of nucleating the helix on raising the pH or, as is suggested, it could be caused by the difficulty of nucleating the adenine and cytosine complex on lowering the pH.

4. The Effect of Potassium Chloride

(a) Shift to Higher Temperature of the O.R. Transition

The O.R. versus temperature curves for kappa-carrageenan (from Chondrus crispus) are shown for various concentrations of potassium chloride in graph 1.6. At all concentrations of carrageenan, the transition is shifted to higher temperature when KCl is added. This is illustrated in graph 1.11 where the points, at which the hysteresis loops were judged to close on heating, are plotted against the concentration of KCl for 0.5, 1.0, 2.0 and 3.0% w/v kappa-carrageenan. Similar curves were obtained for the point where the O.R. transition commenced on cooling (graph 1.12).

Two effects are probably important. The first parallels the behaviour of the transition temperature of DNA, which increases with the logarithm of the salt concentration [88]. This applies generally to salts

and most likely results from suppression of electrostatic repulsions. The ordered conformation of the double helix has a higher charge density than a random coil, thus suppression of charge repulsion will be more important for the ordered species.

The second effect is only seen for specific ions and is thought to be due in part to a requirement for an exact fit of the ion size. Thus K^+ ions gel kappa-carrageenan whereas Na^+ ions do not. This necessity for exact fit of the cation is seen for other polyelectrolyte systems where the polymer chains are aggregating (e.g. alginic acid and calcium [41]). It would therefore seem that K^+ ions are the correct size to fit in sites on the double helix surface and between aggregated helices, thus replacing the electrostatic repulsions of the sulphate groups by electrostatic attraction.

Aggregation of the chains is facilitated in the presence of K^+ ions since potassium salts of sulphuric esters are in general more easily desolvated than sodium salts,[89] because of the radius ratio effect.[90] Specific effects could also arise from the lower requirement of K^+ ions, compared with Na^+ ions, for an exact geometry of the oxygens in the co-ordination complex.[90]

(b) Decrease in the Breadth of the O.R. Transition

Examination of graph 1.6 reveals that addition of KCl sharpens the transition. Similar effects with addition of salt have been observed for DNA. The reasons that Dove and Davidson [88] advance for changes in the transition breadth apply generally and arise from at least two sources:

(i) differing magnitude of shift on the various species of a polydisperse material is very likely to be involved in carrageenans because any difference in transition temperature between molecules of different sulphate content would tend to be suppressed by addition of KCl;

(ii) a change in the co-operativity of individual double helix to coil transitions.

(c) Effect on Gel Formation, Turbidity and Strength

Gel formation depends on the number of junction zones present, turbidity and gel strength on both their number and nature. In carrageenan gels junctions are of two types, double helices and aggregated double helices. Both double helix formation and aggregation are increased by KCl addition. Thus in a particular kappa-carrageenan solution, the double helical crosslinks that are necessary for gel formation will form at a higher temperature in 1.0% w/v KCl solution than in water. This is shown (Table 1.1) by the fact that KCl addition raises the melting and setting points of a gel. Graph 1.6, however, shows that gel formation does not always occur at the same conversion to double helix as judged by the rise in O.R. KCl must either increase the contribution to crosslinking from some other source (viz. aggregation) or form a greater number of shorter double helices. An increase in aggregation is obvious from the increasing turbidity when KCl is added, and indeed produces difficulties from O.R. artifacts at higher temperature and lower polymer concentration than in aqueous solution.

This increase in the number of junction zones, under a given set of conditions, also explains the reported increase in gel strength when KCl is added to kappa-carrageenan solutions.[91]

(d) Increase in Hysteresis

The fact that hysteresis increases as KCl is added must mean that the increased ease of helix nucleation is more than offset by stabilisation of the aggregated helix.

5. Thermodynamic Considerations

By consideration of the heating (equilibrium) curve for the O.R. versus temperature behaviour of segmented kappa-carrageenan it should be possible to calculate the Van't Hoff heat of the transition and also obtain some information on the nature of the transition such as the number of double helices in an aggregate. If the change is written

Aggregated helices $\rightleftharpoons$ 2n Coils, from the Van't Hoff isochore

$$\frac{d \ln K}{dT} = \frac{\Delta H^{o}}{RT^{2}},$$

in which the equilibrium constant $K = \frac{[\text{Coil}]^{2n}}{[\text{Aggregate}]}$ and ΔH^{o} is the Van't Hoff heat, by integrating we have

$\log K = -\frac{\Delta H^{o}}{2.303\ RT}$ + constant. Thus a plot of log K vs. 1/T will give a straight line and ΔH^{o} can be obtained from the slope. Plots of log K vs. 1/T can be obtained in two ways:

(i) K may be evaluated for several temperature steps during a transition, from the ratio of helix to coil as given by the O.R. shift at that temperature;

(ii) at the midpoint of the transition (Tm) K can be expressed as a function of total polymer concentration for several different polymer concentrations.

In either case, n is evaluated by finding the best fit to give a straight line from log K vs. 1/T. Unfortunately, such calculations were completely inconclusive as n = 1 or n = 100 gave almost equally good fits. This probably reflects the insensitivity of the method when applied to a system such as this which shows polydispersity in chemical structure, molecular weight and aggregate size, all or any of which invalidates such treatment. Such calculations have, however, been successfully applied to the coil to triple helix conversion of a monodisperse peptide from collagen and some idea of the forces involved has been obtained from ΔH°. [74]

Helix-coil transitions of high molecular weight material can show the characteristics of a phase change. It has been shown [92] that the equilibrium constant for a two state transition changes more rapidly with temperature the higher the enthalpy change per co-operative unit. The enthalpy change for the transition is of course proportional to the molecular weight.

In collagen, where the change of enthalpy is about 3000 K cal/mole of trimer, the transformation has been considered as a phase change on account of the extreme temperature dependence of the equilibrium constant which overshadows any concentration dependence.[80] Piez and Sherman [74] have obtained a monodisperse peptide from collagen, which has only 36 residues compared with the 1000 of the undegraded molecule. The enthalpy change for this transition is thus about 100 K cal/mole and as a result the transition is not particularly sharp and is concentration dependent.

The helix-coil transitions of iota-carrageenan, kappa-carrageenan and segmented iota-[93] and kappa-carrageenan all show a marked dependence on concentration indicating that the enthalpy change of the transformation, and hence the number of residues involved in each helix, is relatively low. Although these reactions are co-operative they are probably not sharp enough to merit consideration as phase changes.

The transition point for agarose, however, shows concentration independence, [81] suggesting that the process has some phase change character even though the calorimetric heat per residue is similar to that for iota- and kappa-carrageenan. [82] This supports the idea that the phase change here is not helix-coil but aggregated helix-coil. The enthalpy change for the formation of an aggregate of n helices will be at least n times that for the simple helix-coil transition. This characteristic of a phase change is also shown by kappa-carrageenan transitions in concentrated KCl solution (i.e. where the chains are highly aggregated). This is demonstrated by the decreasing sensitivity of the transition temperature to concentration as more KCl is added (graphs 1.13, 1.14).

6. Kinetic Considerations

Here again polydispersity of the carrageenan systems must complicate the analysis, although analogy with other biological macromolecules can give some interesting insights.

In a system in which a coil to double helix transition is uncomplicated by aggregation we would expect nucleation of the double helix to be rate limiting and hence the reaction would be expected to show second order kinetics at the outset. However preliminary examination of the rate of the transition in kappa-carrageenan solutions shows that sophisticated instrumentation would be essential to follow the reaction as it is so fast.

The mono-disperse collagen peptides [74] and DNA [79] have been shown to obey third order and second order kinetics respectively in the initial rate of helix formation.

7. Family Trends and the Role of Aggregation in Gelation

The family shown in Table 2 includes two kappa-carrageenans, iota-carrageenan, kappa-furcellaran and agarose. Various features of the gels of these materials are also listed. The explanation of these family trends has been known for some years to reside in their varying sulphate content. Increasing sulphate content has, as stated earlier, two effects which are both due to electrostatic repulsions and related factors of the sulphate groups. The first is a tendency to resist the formation of the double helix and the second a tendency to resist subsequent aggregation of these helices. The influence of sulphate on the helix to coil transition is demonstrated by the fact that at low concentrations 0.1% w/v agarose chains can come together and undergo a coil to double helix transition, whereas no transition can be detected for kappa-carrageenan under equivalent conditions. The influence of sulphate on aggregation is demonstrated by the turbid nature of agarose gels and the corresponding clarity of those of iota-carrageenan.

The effects which were demonstrated above, where the electrostatic repulsions of sulphate groups are suppressed by KCl, are also seen when sulphate is progressively removed from one member to another in the family. Thus the number of junctions (of both types) increases as we go from one polymer to another and this explains the variation in gel strength, rigidity, turbidity and minimum gelling concentration.

Various concentrations of iota-carrageenan gel at roughly the same conversion to double helix, judged by O.R. rise (graph 1.4). This is not true however for kappa-carrageenan (graph 1.1) as double helical cross links are augmented by aggregate junctions to an increasing extent as the polymer concentration is raised. Aggregate junctions probably also contribute significantly to the properties of kappa-furcellaran and agarose gels.

Syneresis is brought about by contraction of the polymer network squeezing water out of the gel.[55] Thus syneresis also follows the decreasing sulphate trend. In iota-carrageenan gels and kappa-carrageenan gels (without added KCl), where the sulphate groups prevent helix aggregation, no syneresis is observed.

Lambda-carrageenan does not form gels and shows no sharp change in O.R. with temperature (graph 1.5). This is due to the fact that double helix formation is impossible, even after conversion of the 4-linked residue to the anhydride, because 2-sulphation of the 3-linked residue blocks the interchain hydrogen bond.

8. Comparison with a Mucopolysaccharide

Several years ago the structural similarities between carrageenans and glycosaminoglycans, especially hyaluronic acid (fig. 1.19), led to the suggestion that these molecules could also adopt a double helical conformation. [63] This analogy was underlined by

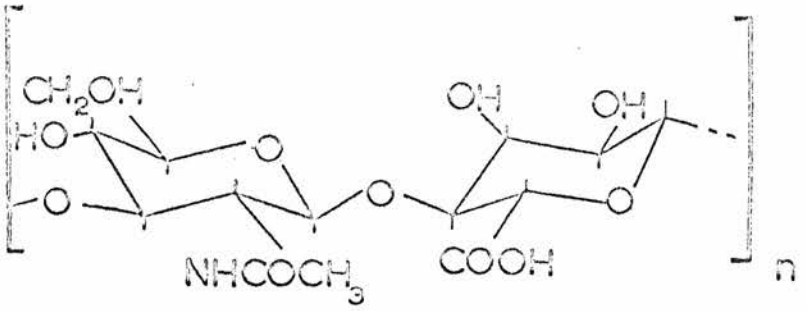


Fig. 1.19. Hyaluronic acid.

computer model building. Recently X-ray diffraction studies on stretched films prepared from a viscoelastic putty of hyaluronic acid have provided evidence for the existence of such double helices. [44] The ability of hyaluronic acid solutions to yield to slowly applied stress and to resist rapid application of stress has been interpreted in terms of a model which envisages that hyaluronic acid molecules in solution can consist partly of double helical regions and partly of random coil regions. [44] The two forms can interconvert under normal

conditions and it is the rate of this interconversion which governs the reaction of this system to mechanical stress. If the application is more rapid than the molecular rearrangement allowed by the coil to helix transition the system acts like a permanent gel but if time for rearrangement is allowed the shape of the system can be easily changed. This behaviour could be regarded as time dependent hysteresis, with difficulty of helix nucleation presenting a kinetic barrier.

These properties can explain the varying biological roles of hyaluronic acid e.g. action as a lubricant which depends on the viscosity (slowly changing systems) and action as a gel-like cushion in joints where sudden increases in stress are experienced.

Considering the fact that a volume contraction is known to accompany the coil to helix transition of carrageenans [62] it does not seem impossible that hyaluronic acid solutions under pressure (as in synovial sacs) could undergo a pressure driven coil to helix transition.

9. Structure and Function of Carrageenan

Much is now known about the chemical structure and molecular conformation of the carrageenans, and over the last few years it has been possible to appreciate how biological properties are modified by changes in the shape of the chain, which are brought about by variation in the primary structure.

Lambda- and xi-carrageenan cannot form double helices as they are 2-sulphated on the 3-linked residue and have no 4-linked 3,6-anhydro residues. Thus these components, although highly viscous, do not gel and must be present to modify the gel set up by other components.

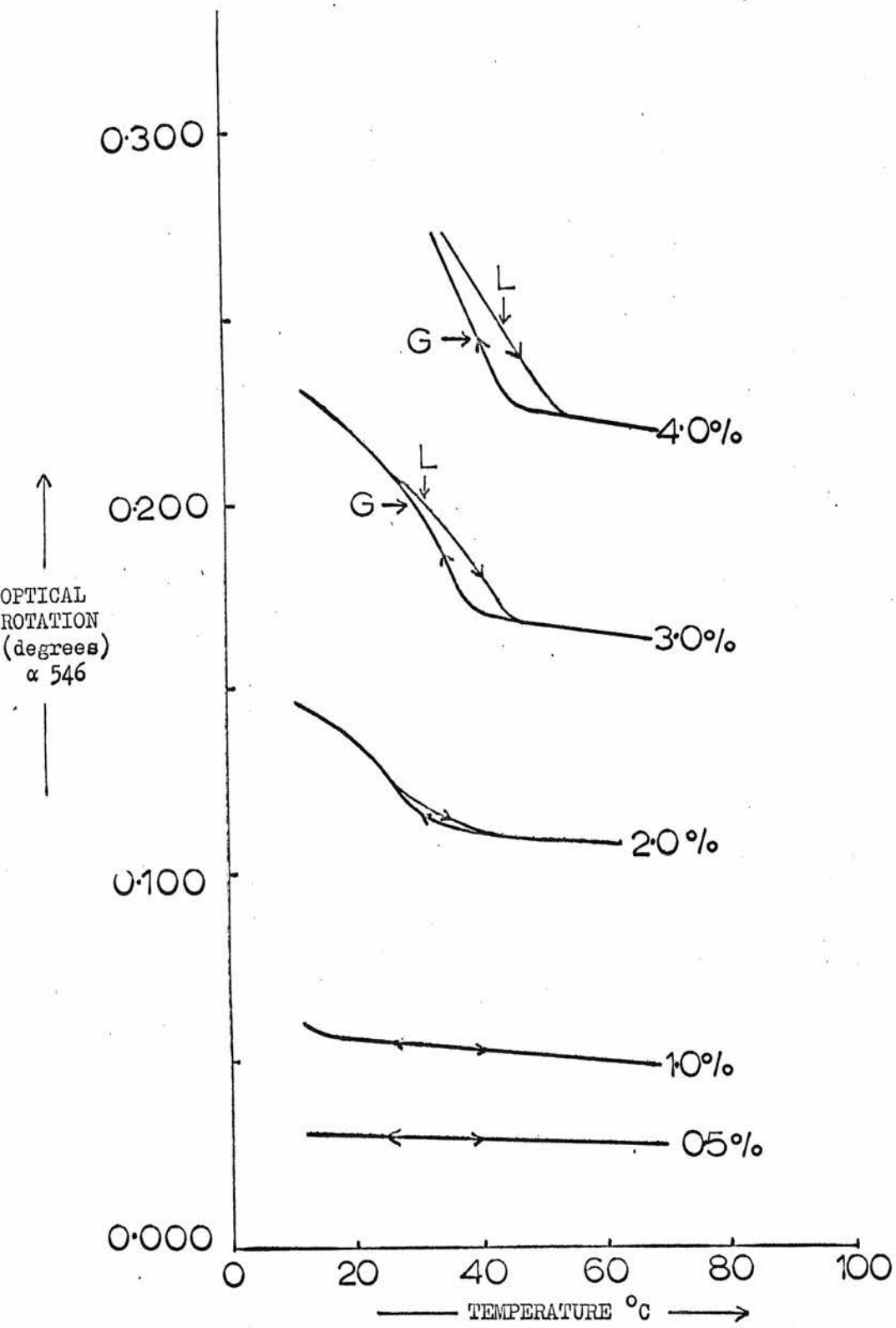
Mu and nu-carrageenan also contain very little 3,6-anhydro 4-linked residues and thus cannot gel but the plant can apparently convert them to kappa- and iota-carrageenan respectively. [57]

The gel formed by the carrageenans must function under the seasonal weather changes and varying environmental conditions that the plant can encounter. To achieve this the strength and nature of the gel can be altered by fine changes in the primary structure. For example it has been shown, that in exposed areas, the agar-like polysaccharide of one species of Rhodopyceae contains a significantly higher proportion of anhydro residues. [94] This has a profound effect on gel properties and it could be that the 3,6-anhydro-α-D-galactose content is varied enzymically as a means of altering gel properties. [57]

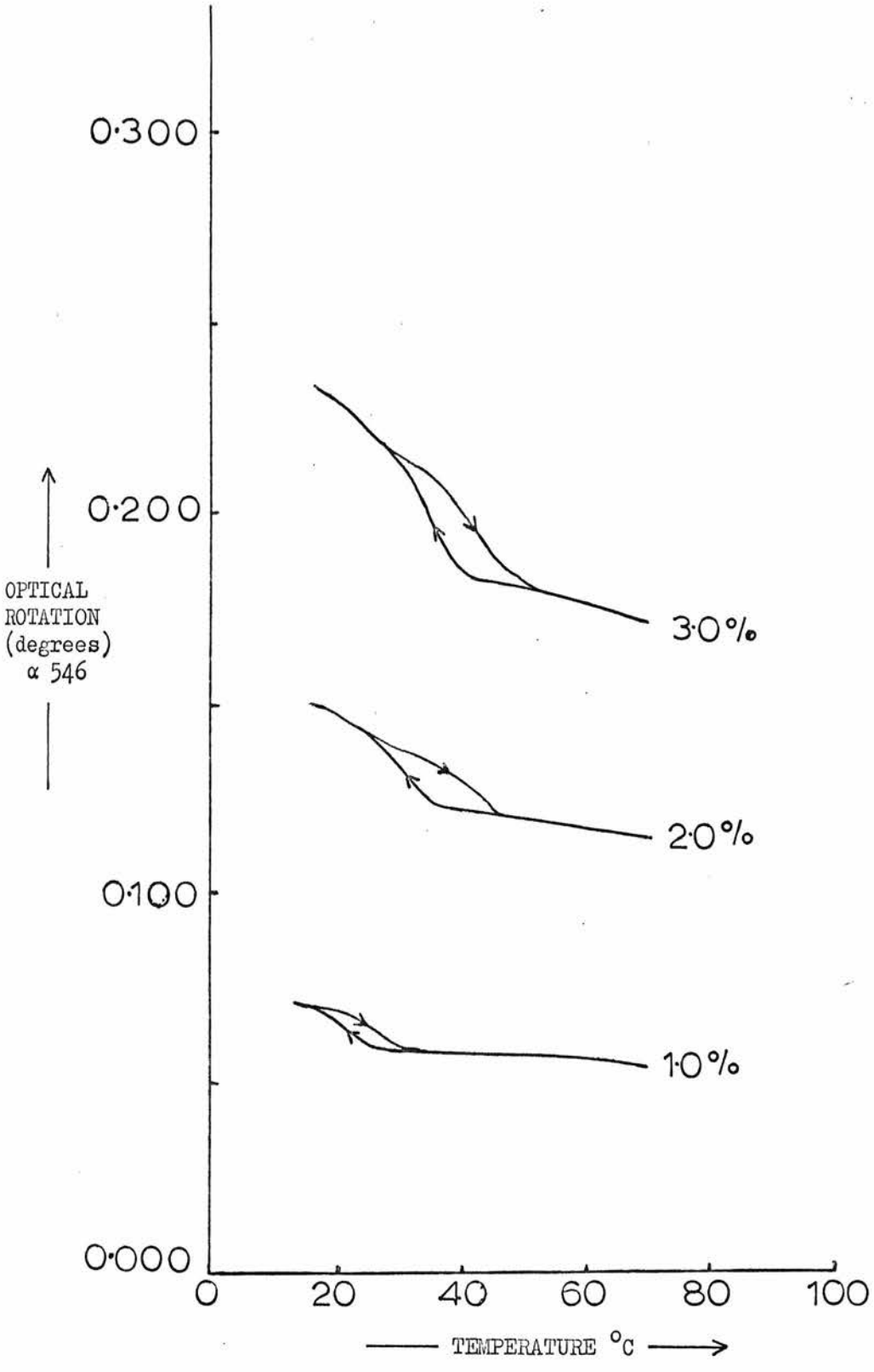
Gross changes could be brought about by variation in the proportions of each type of carrageenan present. The degree of sulphation controls not only structural properties and those of water retention, but also the ability to bind ions both generally and specifically.

The importance of 2-sulphation of the anhydro residue on helix aggregation has been shown in earlier sections. The extent of sulphation of this unit could thus be a source of biological control as gel properties are highly dependent on the degree of aggregation.

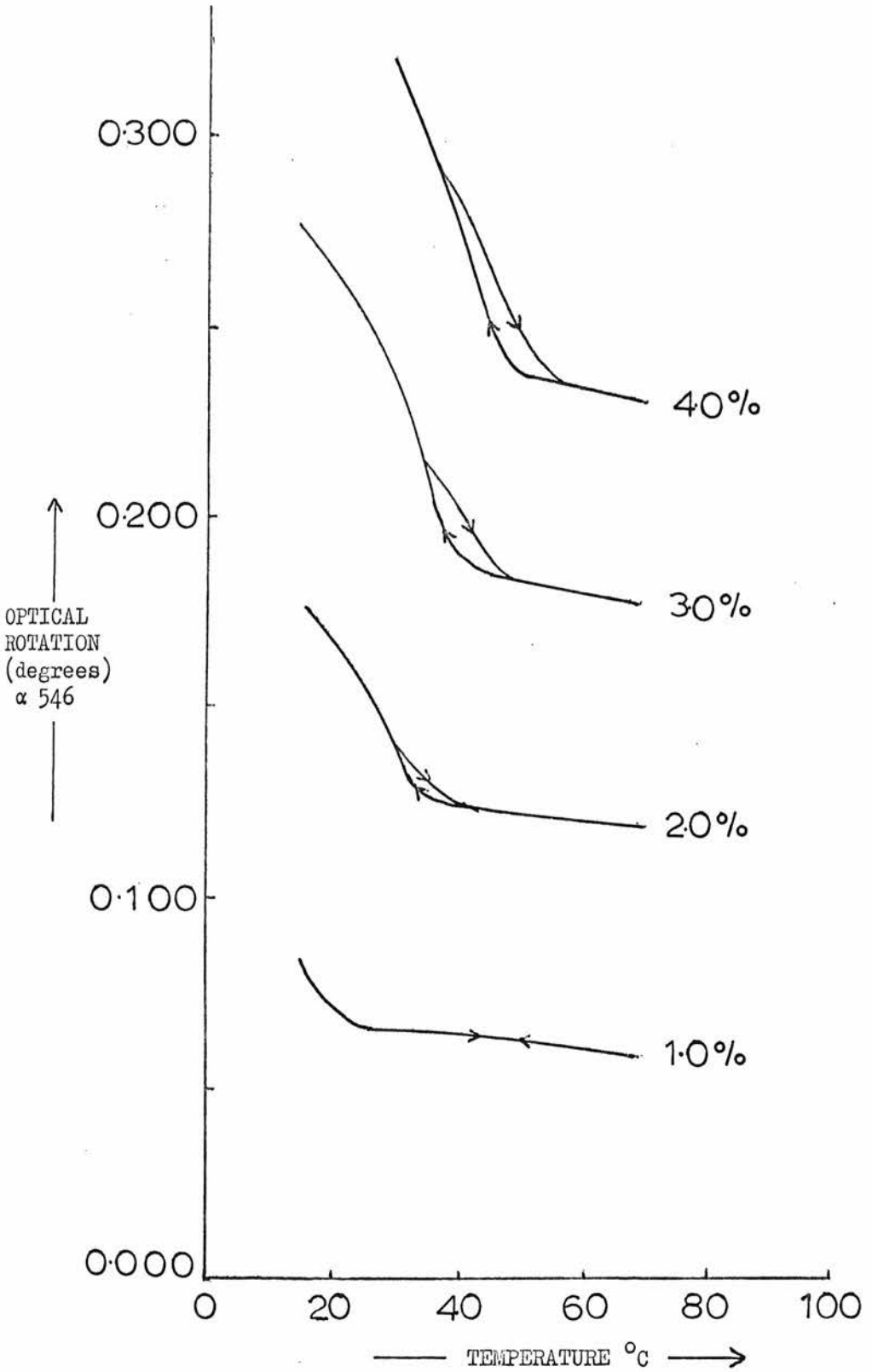
D. GRAPHS AND TABLES



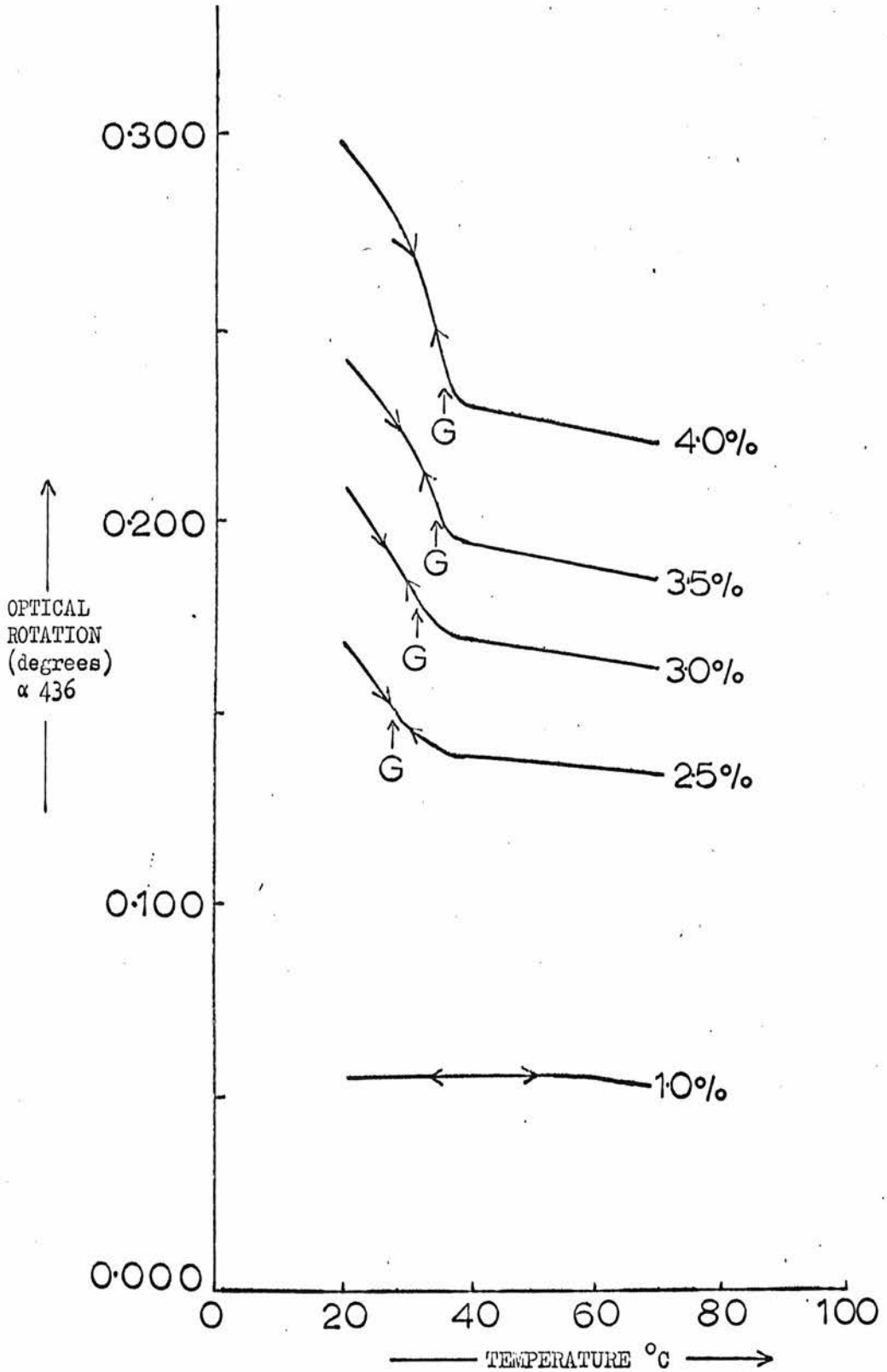
Graph 1.1. Variation in optical rotation on heating and cooling aqueous solutions of kappa-carrageenan from Chondrus crispus. Gel points (G) and liquefaction points (L) are shown.



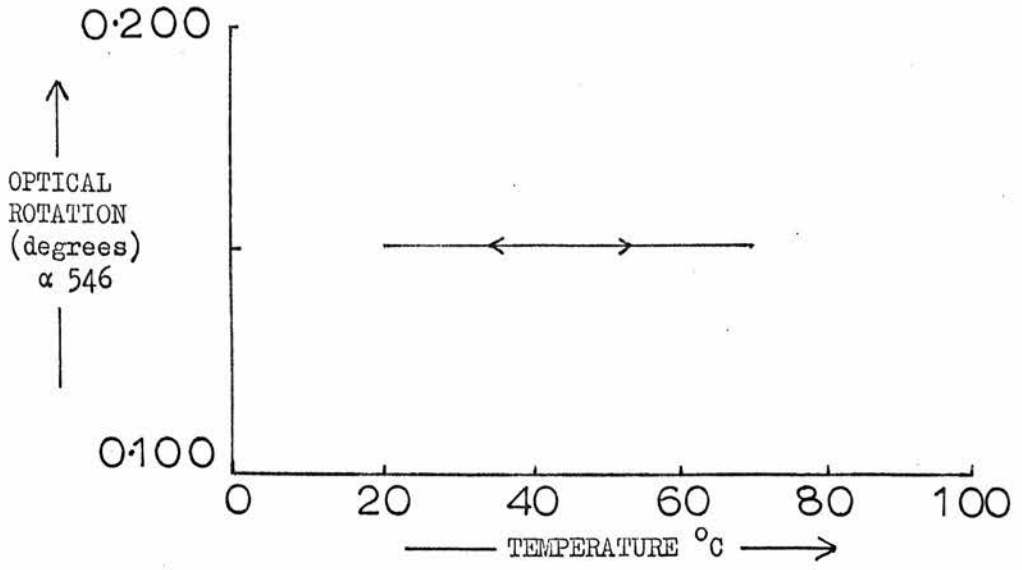
Graph 1.2. Variation in optical rotation on heating and cooling aqueous solutions of kappa-carrageenan from Eucheuma cottonii.



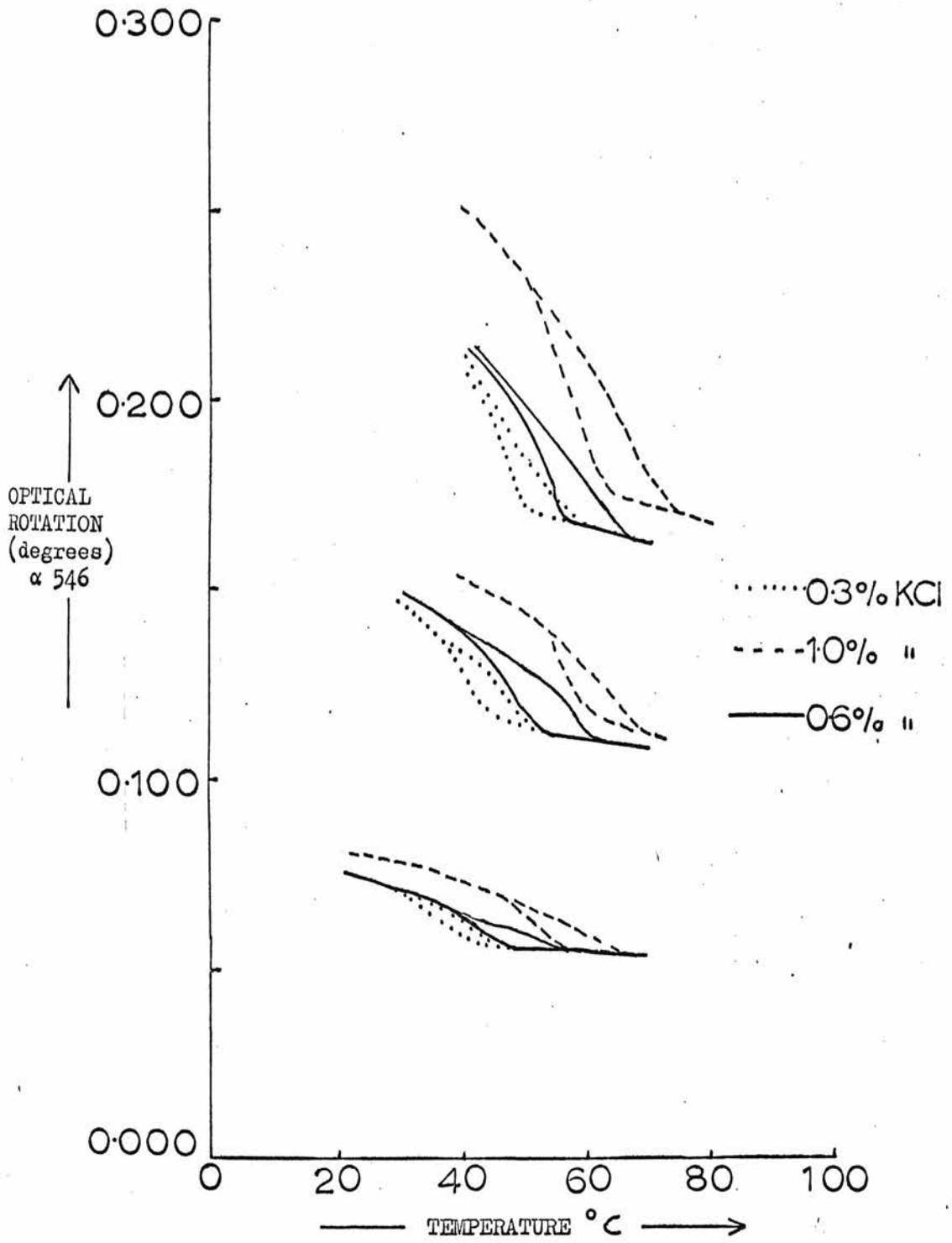
Graph 1.3. Variation in optical rotation on heating and cooling aqueous solutions of segmented kappa-carrageenan.



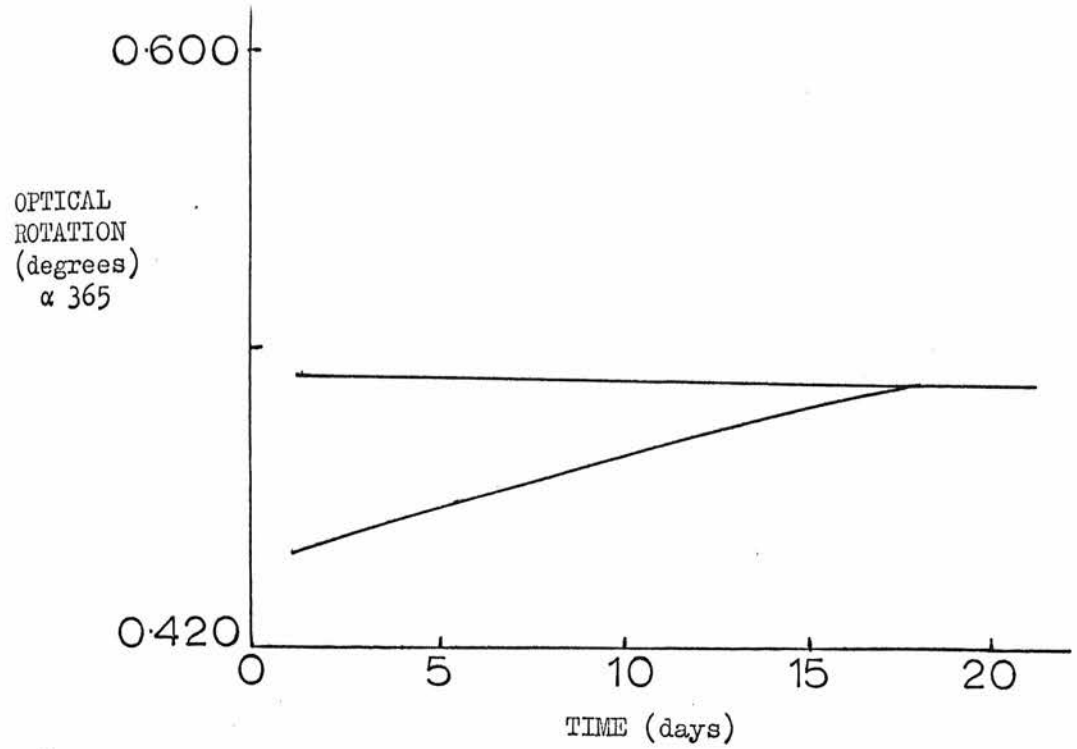
Graph 1.4. Variation in optical rotation on heating and cooling aqueous solutions of iota-carrageenan. Gel points (G) are marked.



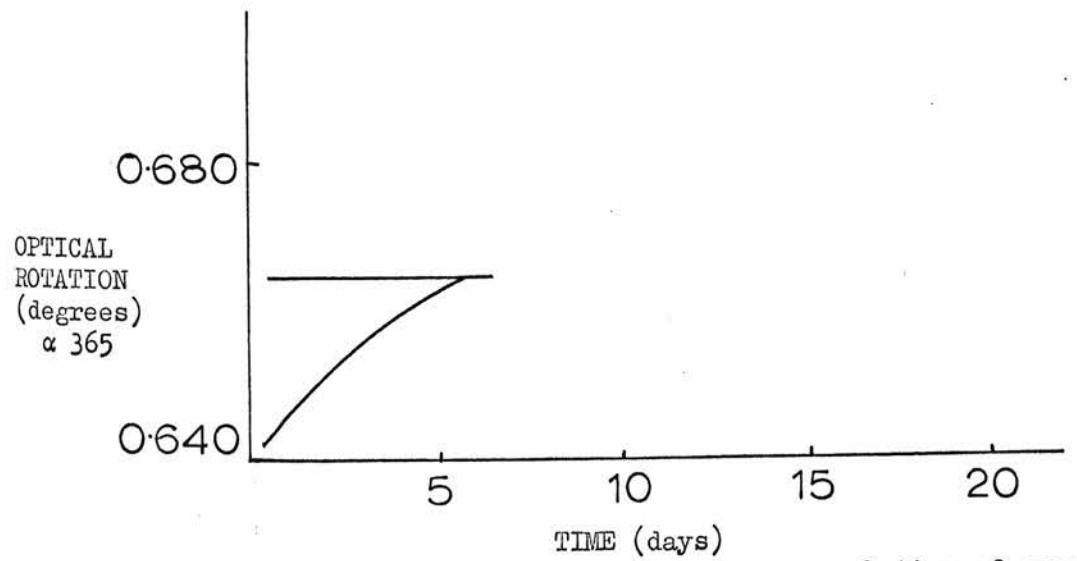
Graph 1.5. Variation in optical rotation on heating and cooling on aqueous solution of lambda-carrageenan (3%).



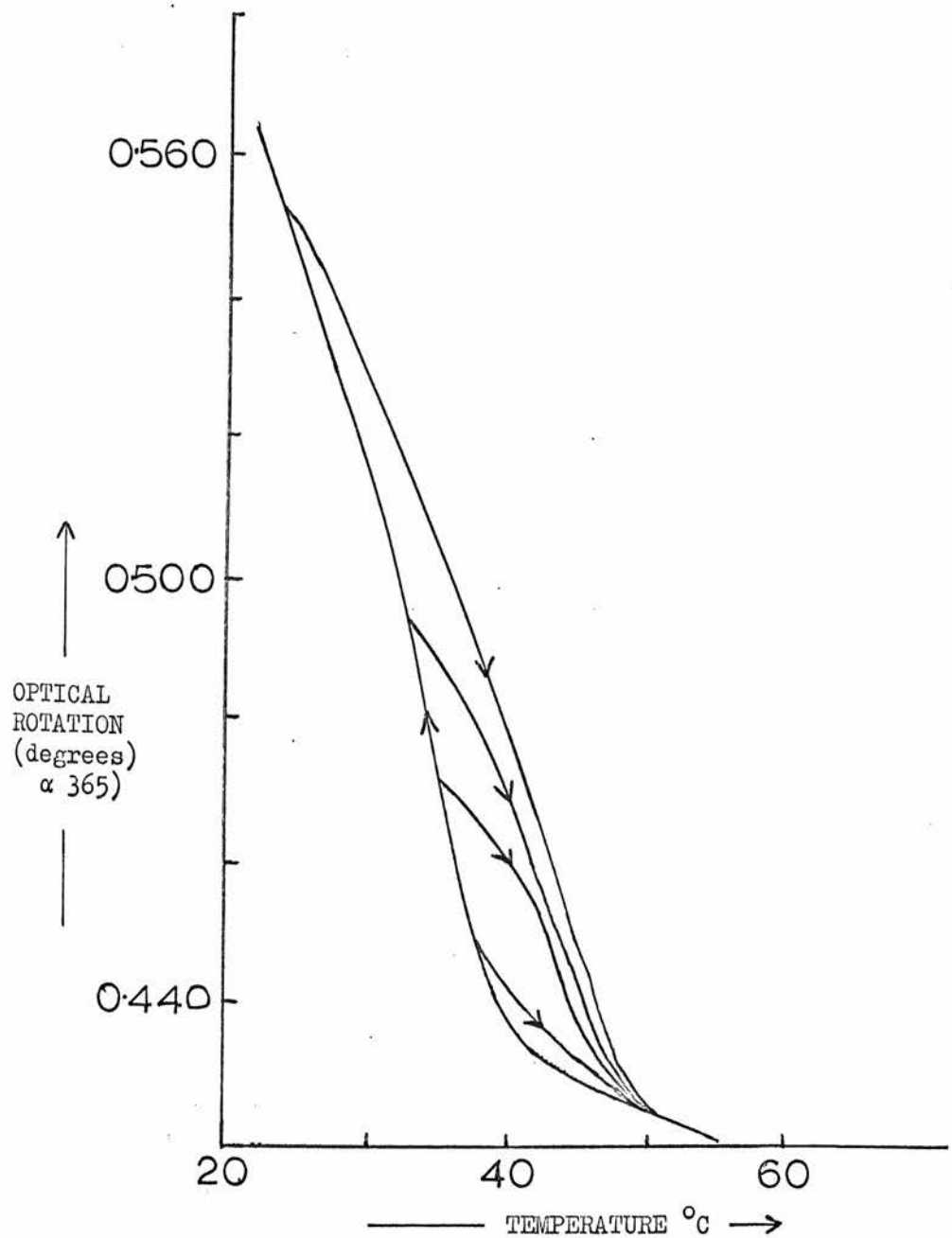
Graph 1.6. Variation in optical rotation on heating and cooling kappa-carrageenan (1,2 and 3%) in 0.3, 0.6 and 1.0% potassium chloride solution.



Graph 1.7. Variation in optical rotation of an aqueous kappa-carrageenan solution (3%) when held at 40°C on the heating and cooling branches of the curve on graph 1.1.

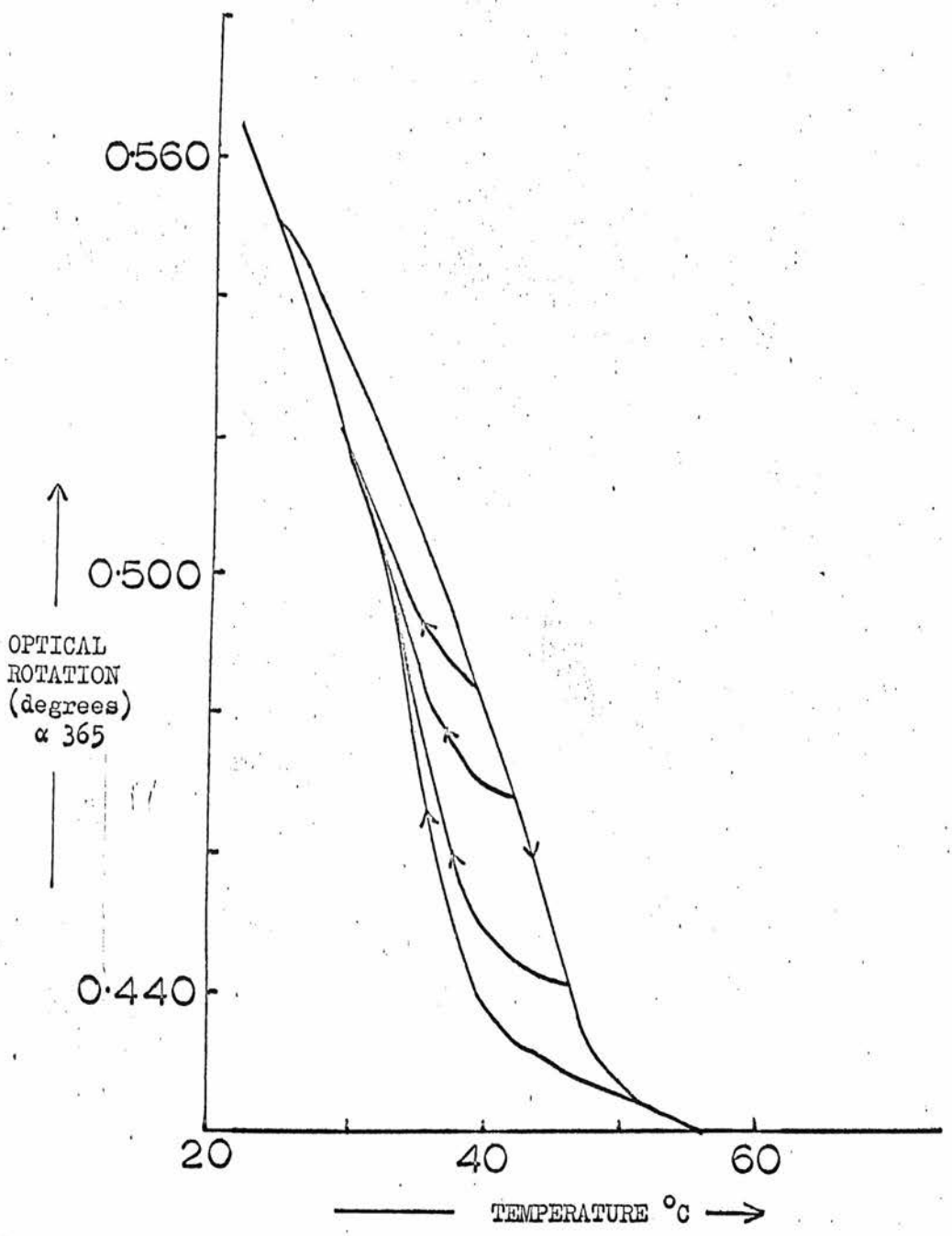


Graph 1.8. Variation in optical rotation of an aqueous solution of segmented kappa-carrageen (4%) when held at 45°C on the heating and cooling branches of the curve on graph 1.3.



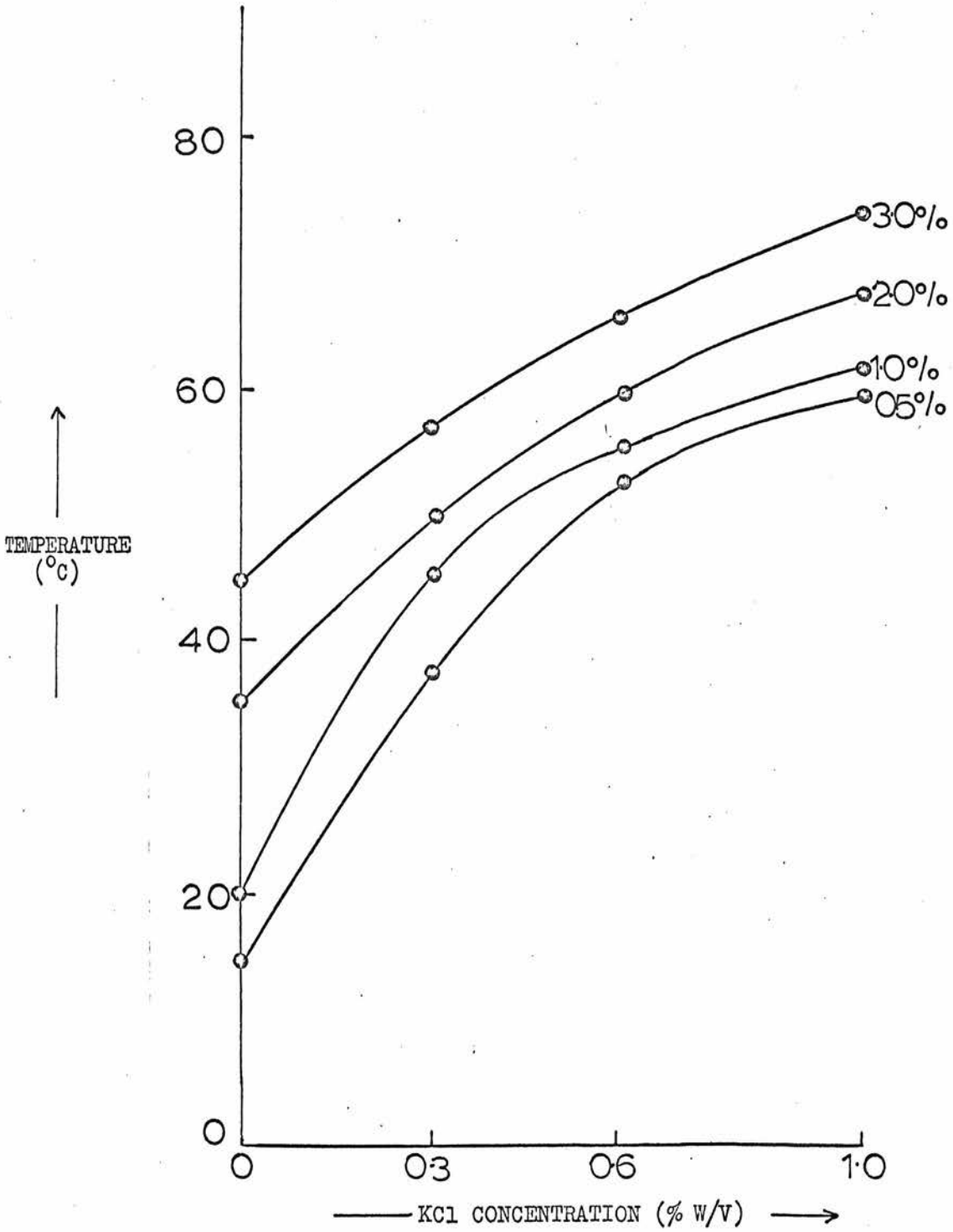
Graph 1.9. Hysteresis behaviour of the variation in optical rotation on heatin

an aqueous solution of kappa-carrageenan (3%). Descending scanning curves.

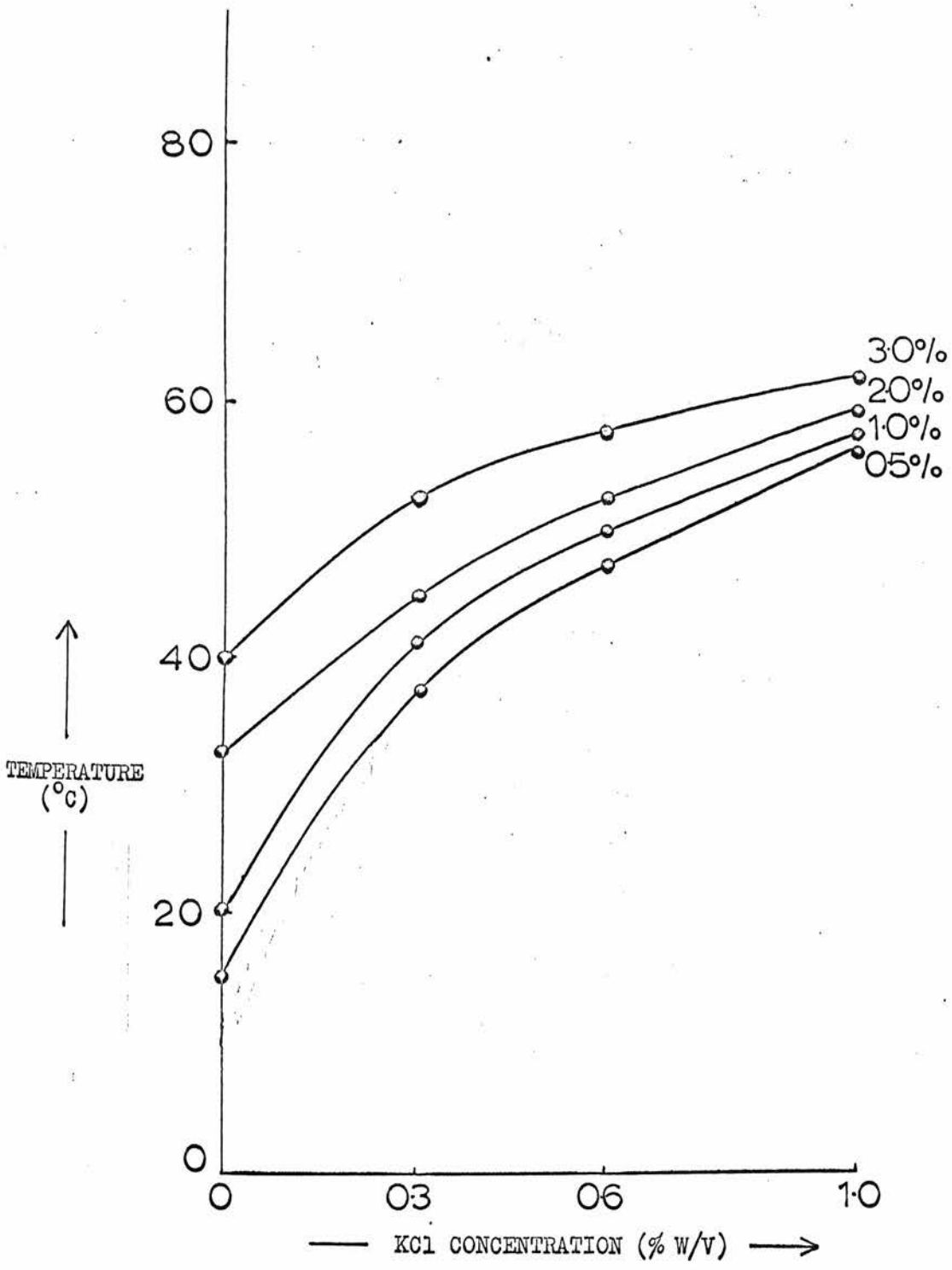


Graph 1.10. Hysteresis behaviour of the variation in optical rotation on heating and cooling an aqueous solution of kappa-carrageenan (3%).

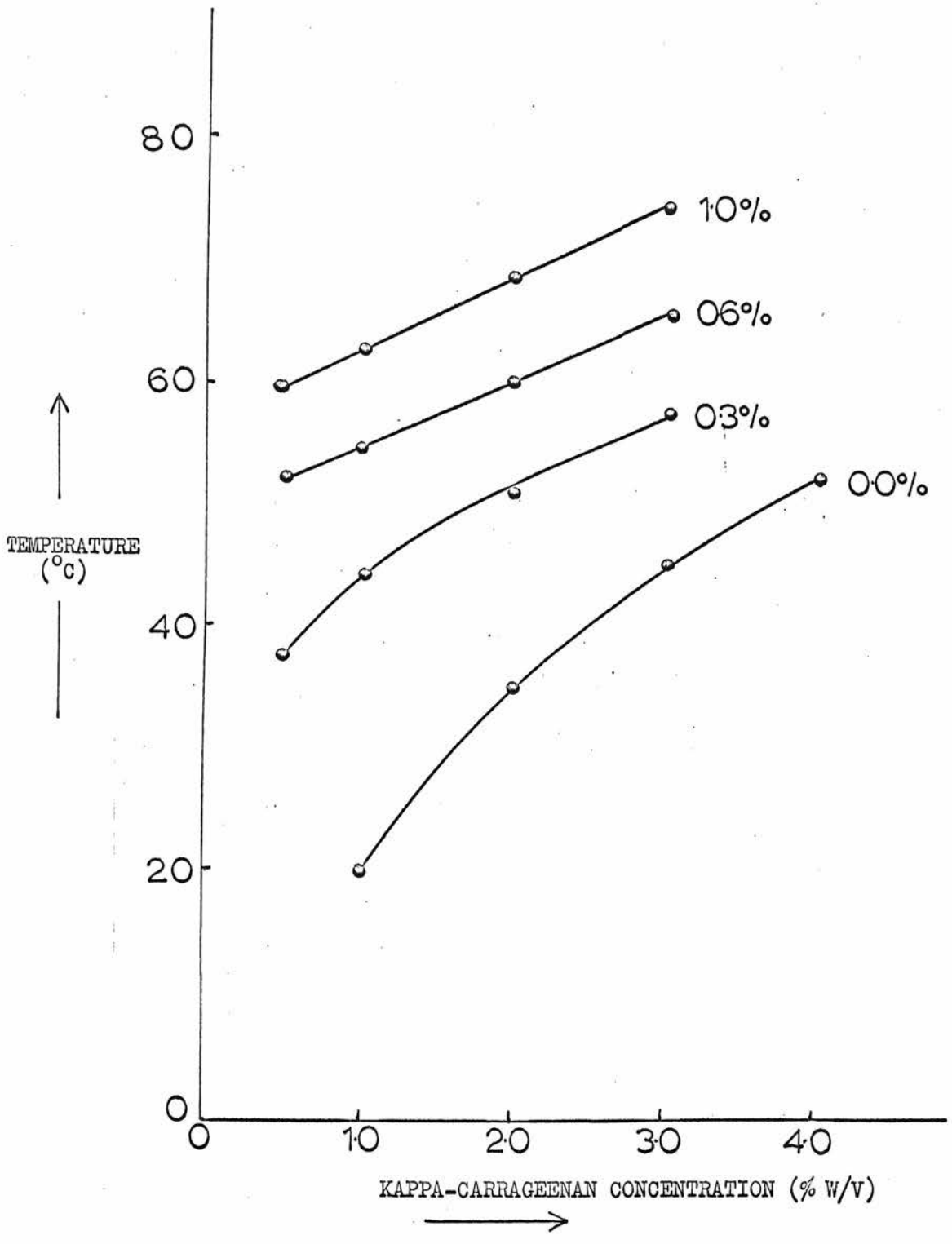
Ascending scanning curves.



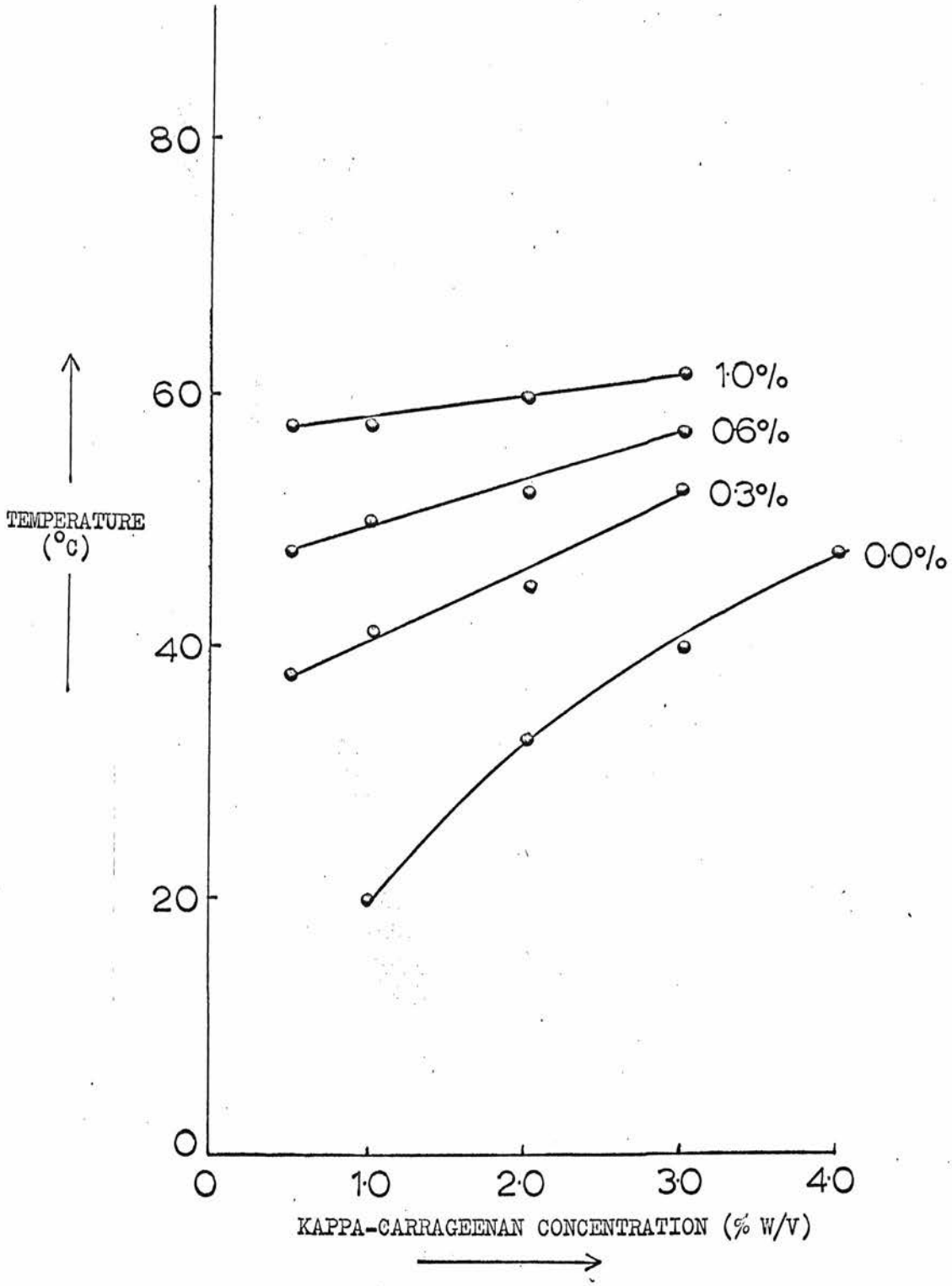
Graph 1.11. Variation in the temperature of the end of the optical rotation transition of kappa-carrageenan solutions with concentration of potassium chloride.



Graph 1.12. Variation in the temperature of the commencement of the optical rotation transition of kappa-carrageenan solutions with concentration of potassium chloride.



Graph 1.13. Variation in the temperature of the end of the optical rotation transition of kappa-carrageenan solutions with polymer concentration in 0.3, 0.6 and 1.0% potassium chloride.



Graph 1.14. Variation in the temperature of the commencement of the optical rotation transition of kappa-carrageenan solutions with polymer concentration in 0.3, 0.6 and 1.0% potassium chloride.

Table 1.1. Temperatures (^{o}C) of gelation (G) and liquefaction (L) of some carrageenan systems.

SOLVENT	KAPPA-CARRAGEENAN (% w/v)					
	2.0		3.0		4.0	
	G	L	G	L	G	L
Water			29	31	39	44
0.3% KCl	37	39	47	53		
0.6% KCl	44	45	52	59		
1.0% KCl	55	58	61	66		

	IOTA-CARRAGEENAN (% w/v)			
	2.5	3.0	3.5	4.0
Water	27	31	34	35

Table 1.2. Dependence of several properties of gels formed from carrageenan or carrageenan-like polysaccharides on the degree of sulphation of the polymer.

	IOTA CARRAGEENAN	KAPPA CARRAGEENAN		KAPPA FURCELLARAN	AGAROSE
		C.crispus.	E.cottonii		
Sulphate half-esters per disaccharide	2	1.3	1.0	0.5	0.0
Magnitude of hysteresis	-	+	++	+++	+++++
Tendency to syneresis	-	+	++	+++	+++++
Rigidity and turbidity of gel	+	++	+++	++++	+++++
Minimum concentration to gel at 25°C, 2°C	2.7, 1.3	2.7, 2.1	1.5, 1.0	0.4, 0.2	0.1, 0.1
Synergistic activity	-	+	++	++	+++++

INTERACTION BETWEEN CARRAGEENANS AND GALACTOMANNANS - POLYSACCHARIDE QUATERNARY STRUCTURE

A. INTRODUCTION

This chapter is concerned mainly with the gelation of mixed polysaccharide systems of carrageenans and galactomannans. The chemistry of the carrageenans has been discussed in Chapter I.

The galactomannans, which are found in the endosperm of the seed of various leguminous plants, form a structurally related family of reserve polysaccharides. The molecules all have a β-1,4-D-mannan backbone substituted by single galactose residues attached α-1,6.[95]

The various members differ in the proportion of mannose residues which carry galactose. It has been suggested [96] that ivory nut mannan, which contains only traces of galactose, may represent one extreme of the group. On the other hand other members, such as fenugreck gum, have been reported which are almost fully substituted.[95] In this thesis the relative proportion of mannose (Man) to galactose (Gal) residues of a galactomannan is shown as the Man/Gal ratio. The galactomannans that have been utilised in this work include:

a) locust bean gum which is obtained from Ceratonia siliqua (Man/Gal ratio of 3.35);

b) tara gum from Caesalpinia spinosa (Man/Gal ratio of 3.00);

c) guar gum from Cyamopsis tetragonolobus (Man/Gal ratio of 1.56);

d) Fenugreek gum from Trigonella foenum-graecum (Man/Gal ratio of 1.08).

The use of locust bean gum has been traced back over many centuries.[97] Paste from the locust bean plant was used by the ancient Egyptians to bind their mummies and the pods of this tree are also thought to be the "bread" that John the Baptist ate in the desert. In fact the fruit of the locust bean, or carob, tree is often used as an animal and human foodstuff.

These carbohydrate polymers, especially locust bean and guar gums, now find use as sizers in the textile industry, thickeners in the food industry and in many other areas.[97]

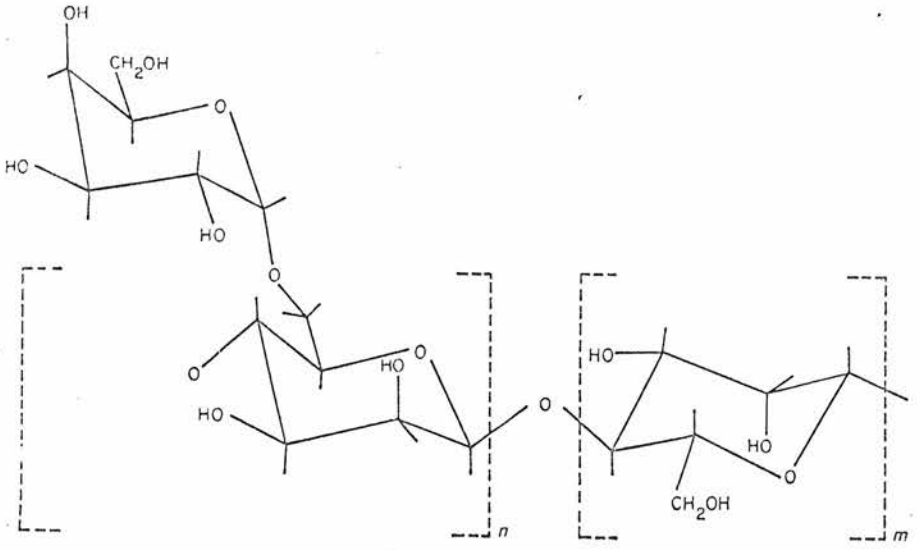
The physical properties of the galactomannans depend to a large extent on their Man/Gal ratio; ivory nut mannan being insoluble whereas fenugreek gum dissolves easily. Guar and locust bean gums, which have molecular weights of the order of 2.5×10^5, form highly viscous solutions. Galactomannans form gels in the presence of borate and guar gum also gels on precipitation with various salts.[98]

It has been shown that locust bean and guar gums have a varying Man/Gal ratio depending on their source.[95] Material from a single source may also be polydisperse as regards this ratio and fractions with varying ratios can be separated on account of their different water solubilities.[99]

Courtois and Le Dizet have proposed [100, 101] that along the β-1,4-mannan chain there are zones in which practically all units are substituted at C(6) with galactose and zones in which the galactose substituents are widely spaced (Fig. 2.1a,b). This proposal is based on the action of β-mannanases on galactomannans from Ceratonia siliqua Man/Gal ratio of 3.88, Gleditschia ferox Man/Gal ratio of 3.9, Trifolium repens Man/Gal ratio of 1.07, and Medicago sativa Man/Gal ratio of 1.1, and on fractions of these materials obtained by partial galactosidic hydrolysis with an α-galactosidase from coffee beans. The authors show that the α-galactosidase acts on isolated galactose stubs and those at the end of substituted regions such as (b) and (e) in Fig. 2.1.

They also show that the β-mannanase is an endoenzyme by the magnitude of the reduction in viscosity after only a few mannoside linkages have been broken. This enzyme did not act on galactomannans which have a Man/Gal ratio close to 1, but in the case of galactomannans with a Man/Gal ratio of 3.5-4.0 it splits off oligomannosides and a non-dialysable product is left which has a Man/Gal ratio close to 1.0. Action of the β-mannanase on all the products which had been subjected to α-galactosidase action produced similar products with Man/Gal ratios about 1.0.

(a)



(b)

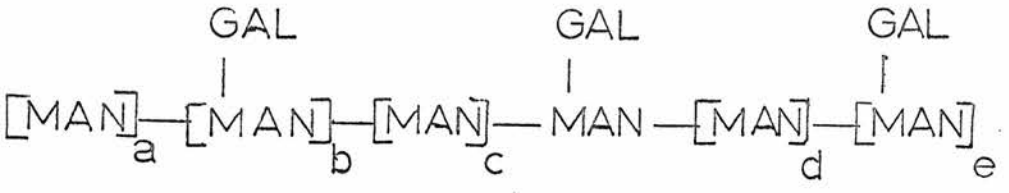


Fig. 2.1 (a), (b) Galactomannan structure showing grouping of galactose stubs.

Chapter I dealt with some aspects of a gelation process which was already largely elucidated but, before going on to discuss gelation of mixed systems about which no ideas of mechanism have been evolved previously, it is worth reviewing some of the general features of polysaccharide gel formation.

Perhaps the most striking feature of polysaccharide gels is the minute quantity of polymeric species that may be required to impart many of the characteristics of a solid to a large volume of water. An amount as little as 1 g of agarose in a litre of water is sufficient to form a gel capable of maintaining the shape in which it is set for a considerable period of time.

It has been shown by steady state and pulsed NMR techniques that the state of the bulk water component in such systems remains unaltered during the formation and melting of several polysaccharide gels.[102 - 104] This is expected from the classical picture of gelation of dilute polymer systems in which the properties of the gel are accounted for by a framework of polymer chains extending throughout the system with water trapped in the interstices.[105] This model demands that the polymer chains immobilise each other by cross-linking. At the molecular level, the nature of the linkage regions, or junction zones, must be elucidated.

Several features of polysaccharide gels have led to the conclusion that the junction zones do not undergo continuous formation and "melting" (i.e. the gel is not in a state of dynamic equilibrium during the period of the experiment).[51]

a) If a gel is cut, and the surfaces held in contact, the gel does not "heal".

b) The network structure of some gels, for example agarose, collapses after a time and some water separates from the contracted network. This reversion to a more stable state would be expected to proceed more rapidly if the junctions holding the chains together were dynamic in the short term.

c) Gels of identical constituents, formed by different processes, can have unlike properties which converge only slowly (e.g. alginate gels).

The "stable" nature of the crosslinks must imply that junction zones cannot be formed entirely by dynamic forces such as isolated ion bridges, single hydrogen bonds and chelation of individual ions.

However gels formed from hyaluronic acid at suitable pH [44] and galactomannan-borate systems show healing ability which could be due to the dynamic nature of the junctions.

In most polysaccharide gels junction zones are formed by the aggregation of relatively long regions of the chains. These aggregates are stabilised by a variety of forces, individually weak and dynamic, which act co-operatively to form stable junctions. On account of the dynamic nature of the individual "links" a junction zone can break up over a prolonged period of time if a more stable link can be formed at the expense of the original.

Molecular organisation of the junction zone has been determined for several polysaccharide gels. In iota-carrageenan under some conditions it appears that only two chains are present in each junction [Fig. 2.2(a)] [55], whereas in other systems, such as microcrystalline cellulose and tobacco mosaic virus gels, particles with an ordered internal structure (the chain microcrystallites or virus particles) coalesce to form a network [Fig. 2.2(b)]. Many gels have junction zones which are aggregates of a number of molecules between these two extremes, e.g. alginate gels [41] [Fig. 2.2(c)].

The forces involved in junction formation can be of several types. In carrageenan gels the chains of the double helix are held together by hydrogen bonding,[62] Van der Waals and polar interactions [106] and possibly ion binding. These helices can in turn be held together by a similar combination of forces.

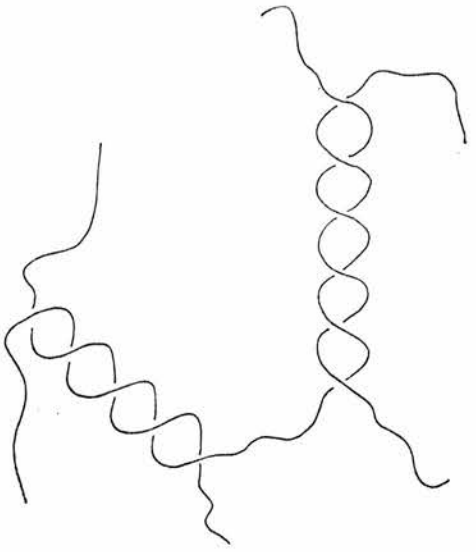


Fig. 2.2(a). Molecular organisation of an iota-carrageenan gel.

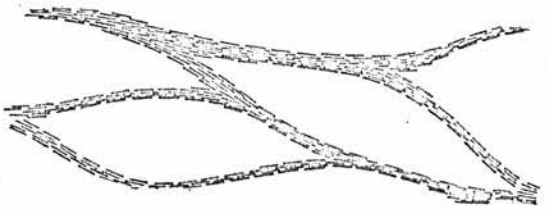


Fig. 2.2(b). Organisation of the virus particles in a Tobacco Mosaic Virus gel.

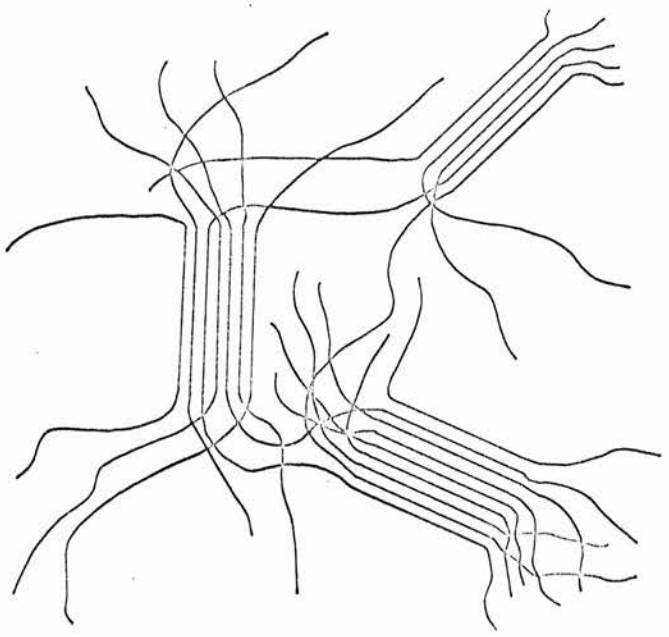


Fig. 2.2.(c). Molecular organisation of an alginate gel.

In the gelation of alginate and pectate, formation of the junction zone has been explained on the basis of the "egg-box model" of chain association.[41] Chain segments group into microcrystallites which are stabilised by co-operative binding of calcium ions sandwiched between the chains (Fig. 2.3).

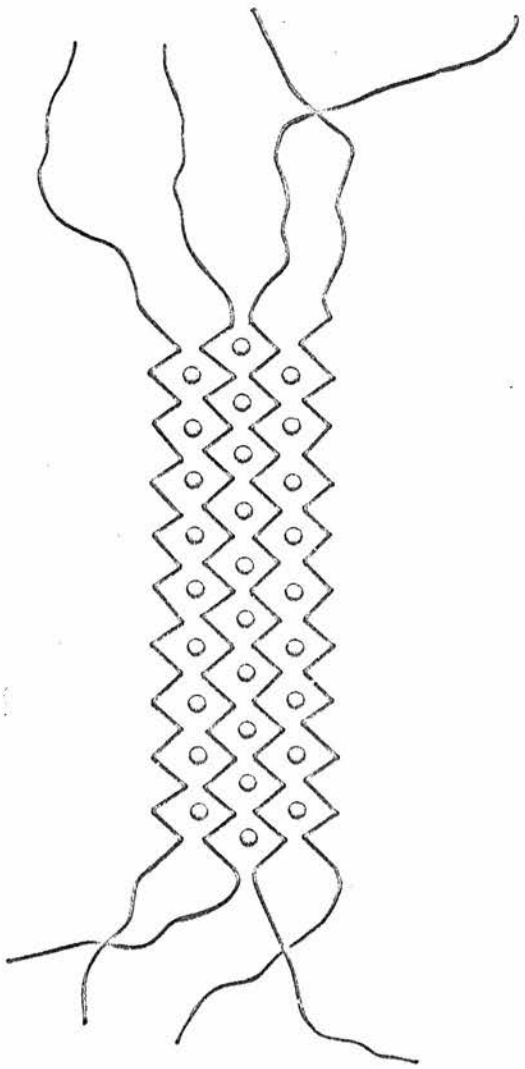


Fig. 2.3. The "egg-box" model for an alginate junction zone.

Entirely different forces dominate in gels of O-methyl cellulose [51]. These gels set on heating and liquify on cooling because the solvating ability of the water decreases on heating. Eventually the water structure around the substituted regions of the polymer molecules is broken down and the chain segments are forced to form micelles which serve as junction zones.

B. EXPERIMENTAL

Materials

These are described in the "General Methods" section. The galactomannans were not dialysed after initial "cleaning" as this was shown to have little effect on the behaviour of these materials. Carboxymethyl cellulose, sapote gum and tamarind seed mucilage were also used undialysed as they did not contain sufficient salts (or counter ions) to bring about gelation of the carrageenan.

Smith-Degraded, Alkali Modified Kappa-Carrageenan and Smith-Degraded Agarose

The preparation of these materials is described in Chapter I.

Fractionation of Locust Bean Gum

Locust bean gum (5 g) was stirred in water at room temperature for several hours. The undissolved material was collected on the centrifuge, resuspended and refluxed in water (1 L) for 1 hour. The residue was collected once again. The two solutions were freeze dried giving 2.2 g and 2.0 g respectively.

Such hot extracts have been shown to have a higher Man/Gal ratio than the original material.[99]

Formation of Mixed Gels

The polysaccharides were dispersed in water then sealed in tubes and heated in a pressure cooker for about 20 minutes. No objective criterion of gel formation was employed, but synergistic activity was judged on the mobility of the cool solutions compared with the individual systems.

Determination of Gel Setting and Melting Points

This was carried out as described in the "General Methods" section. The results are shown on Table 2.1 and on the corresponding O.R. versus temperature plots for the same samples.

Measurement of Optical Rotation versus Change of Temperature

The standard procedure used for these measurements is described in the "General Methods" section. The results for individual carrageenan samples are reproduced in Chapter I. In addition to these

O.R. versus temperature plots were obtained for:

a) locust bean gum (graph 2.4);

b) carboxymethylcellulose (C.M.C.) (graph 2.5);

c) locust bean gum/kappa-carrageenan (Chondrus crispus) mixtures (graph 2.1);

d) locust bean gum/kappa-carrageenan (Eucheuma cottonii) mixtures (graph 2.2)

e) locust bean gum/segmented kappa-carrageenan mixtures (graph 2.3);

f) C.M.C./kappa-carrageenan mixture (Chondrus crispus) (graph 2.6).

Corrections for variation in the refractive index and density of the solvent due to temperature changes were shown to make no appreciable difference to the O.R. measurements and were thus not allowed for.

Ultraviolet and Circular Dichroism Spectra

The methods of recording these spectra and the instrumentation used are described in the "General Methods" section. The results are shown on graphs 2.8-2.12.

Analysis of Galactomannans

These were carried out by Dr. A. Morrison of the Unilever Research Laboratory.

C. RESULTS AND DISCUSSION

1. Synergism

It has been known for many years that the addition of certain galactomannan gums, notably locust bean gum, has a profound effect on the properties of kappa-carrageenan and agarose gels and solutions.[95,97,98] If a carrageenan solution is diluted to an extent where it will no longer gel, subsequent addition of locust bean gum is found to gel the mixture. Addition of galactomannan to gelling kappa-carrageenan systems has several effects:

a) the gel obtained is very elastic, unlike the brittle gel obtained from the carrageenan alone;

b) the gel strength increases dramatically;

c) syneresis is greatly reduced;

d) very clear gels are obtained.

The effects observed on the addition of galactomannan are not expected from simple addition of the properties of the separate components. They have been termed synergistic effects and the phenomenon is known as synergism.

2. Formulation of the Problem

The problem of understanding synergism on a molecular basis is one of determining how mixed gels form. The general features of gelation of only one polysaccharide still hold. Three mechanisms of gelation are therefore likely.

a) The gel network could be set up by the carrageenan molecules alone; the galactomannan having a passive role but somehow increasing the number of carrageenan junctions, double helix or aggregate.

b) The carrageenan molecules could be present in essentially the same state as they occur in the absence of galactomannan, synergism arising from further crosslinking of the carrageenan molecules by galactomannan chains.

c) The carrageenan and/or the galactomannan chains could form junction zones which do not involve the carrageenan double helix. This could lead to the setting up of a gel network of carrageenan chains, galactomannan chains or a mixture of both.

Gelation by type (a) or (c) mechanisms could be brought about by a decrease in the activity of water compared with that experienced in the individual polysaccharide systems. As in the case of pectin-sugar

gels,[51] this would cause more weakly solvated polymer chains to separate from solution and form junction zones which, because of the energy gained by solvation, were unfavourable in the absence of the added component.

The evidence presented and discussed in this chapter indicates that the likely molecular model for synergistic gels is of type (b) with galactose free regions of the galactomannan chains binding to the ordered carrageenan conformation.

Proof of this model rests on establishing several important points:

i) the presence of the carrageenan double helix to a similar extent as would be observed in the absence of galactomannan;

ii) the inability of one component of the synergistic pair to cause gelation on its own;

iii) the involvement of the carrageenan double helix in junction zones with galactomannans;

iv) the involvement of unsubstituted mannan regions in the junction zone;

v) the nature of the junction zone.

3. The Presence of the Double Helix in Synergistic Gels

Several techniques have indicated the presence of the carrageenan double helix in such systems.

a) Gel Formation

Mixed gels of kappa-carrageenan and galactomannan were formed by heating a dispersion of the two polysaccharides in water until dissolved, then cooling. A gel could also be formed by mixing separate solutions of the components at room temperature. Non-gelling concentrations of carrageenan (1% w/v) could be used to form such gels. Locust bean gum and segmented kappa-carrageenan solutions also show this behaviour.

The involvement of the double helix is suggested by the setting and melting points of mixed gels containing gelling concentrations of kappa-carrageenan since these are always close to the melting point of the corresponding carrageenan gel.

The characteristic hysteresis in the melting and setting of kappa-carrageenan gels is retained in synergistic systems. (Table 2.1). Since hysteresis has been shown to arise from aggregation of the carrageenan double helix it is probable that these helices remain in the mixed systems and are responsible for the hysteresis observed.

b) Optical Rotation Changes on Heating and Cooling Solutions of Kappa-Carrageenan and Locust Bean Gum

The O.R. behaviour of kappa-carrageenan solutions was discussed in Chapter I. A typical O.R. versus temperature curve for locust bean gum is shown in Graph 2.4. The O.R. measurements on mixed systems were carried out on the two kappa-carrageenans and the degraded material studied in Chapter I. The results are shown on Graphs 2.1, 2.2 and 2.3. An example of the curve obtained by addition of the O.R. behaviour of the individual systems is shown for comparison. In all cases it is found that the O.R. versus temperature behaviour of the mixed system and that predicted by addition of the behaviour of the separate systems are almost identical. Since this is observed at several concentrations of locust bean gum and carrageenan for three carrageenan samples it seems that these O.R. changes almost certainly arise from the coil to double helix transition of the carrageenan.

Hysteresis of the O.R./temperature curve is not only retained in mixed systems but it also occurs over roughly the same part of the transition. The relative magnitude of the hysteresis loops for the three samples is also retained.

The presence of the normal carrageenan helix-coil transition can be inferred, not only from the transition itself, but also from the dependence of this transition on carrageenan concentration. The

dependence of both the commencement of the O.R. transition on cooling and the end of this transition on heating show roughly the same dependence on carrageenan concentration regardless of the presence of galactomannan. This is shown for the segmented material in Graph 2.7.

c) X-Ray Fibre Diffraction

Fibres prepared by gentle drying of gels of 3% kappa-carrageenan (Chondrus crispus) and 1% locust bean gum gave X-ray diffraction photographs which appear to be unchanged relative to those obtained from kappa-carrageenan fibres.[81(a)] This indicates that the double helix is still formed in the synergistic fibre. The methods used in the X-ray diffraction work were essentially as described by Anderson et al for kappa- and iota- carrageenan.[61]

d) Reporter Molecules

Methylene blue has been suggested as a conformational probe for carrageenans.[62] When the dye is present in solution with

carrageenan molecules in the random coil conformation (at high temperature) the ultraviolet spectrum obtained is essentially the same as that of the dye alone (Graph 2.8 and 2.9). At high temperature in the presence of carrageenan or at any temperature without carrageenan no circular dichroism spectrum is obtained from the dye. If the dye is present in systems where carrageenan molecules are in the helical conformation there is a pronounced shift in the population of the ultraviolet peaks (Graph 2.9) and a circular dichroism spectrum is induced (Graph 2.10). This characteristic behaviour is retained in synergistic systems with locust bean gum (Graphs 2.11, 2.12).

The use of methylene blue and other aromatic dyes as a quantative probe for carrageenan conformation is, however, severely limited by the stacking tendency of these dyes. This is discussed in Section 12 of this chapter.

4. The Possibility of One Component being Responsible for the Gel Network

a) The Carrageenan Component

The carrageenan component could set up a gel network on its own if the galactomannan brought about an increase in double helix

junctions, aggregate junctions or caused the formation of some completely new junction.

The main evidence against such a mechanism comes from the gels formed by adding locust bean gum to carrageenan and agarose segments. In carrageenan systems at low temperature (25°C) there is no O.R. evidence of an increase in the helical nature of segmented or undegraded material on the addition of galactomannan (Graphs 2.1-2.3). It could be argued that the polymer forms a greater number of smaller double helix junctions but the co-operative nature of the process makes this unlikely. The clarity of synergistic gels formed from segmented kappa-carrageenan and locust bean gum suggests that the gel is not formed by "virus fashion" micelle formation (see Introduction). This is underlined by the behaviour of agarose segments. As stated in Chapter I, when a solution of agarose segments is cooled the molecules aggregate and precipitate from solution but in the presence of galactomannan the mixture was found to gel, indicating that, if anything, aggregation is reduced. Periodate oxidised and enzyme degraded locust bean gum (see later) do not bring about gelation of non-gelling carrageenan systems.

b) The Galactomannan Component

Several factors indicate that the galactomannan component is not solely responsible for the gel network.

i) At the concentrations and under the conditions used in the promotion of synergistic gels, galactomannan solutions show no gel character.

ii) If the gel resulted from such a network it is difficult to envisage why synergistic gel formation should show the specificity for the non-galactomannan component that it does. It appears that synergism is only observed for agarose, kappa-furcellaran and kappa-carrageenan. No synergistic activity could be detected with the highly sulphated iota-carrageenan. Gelation could not be brought about by addition of equivalent concentrations of dextran, laminaran or amylose to locust bean gum. Gelation by a network of galactomannan chains alone does not explain the vast difference in synergistic activity shown by agarose and carrageenan systems.

iii) The temperature of gelation in each system suggests that the ordered carrageenan conformation is involved in gelation. In all cases O.R. measurements coupled with gel point determinations indicate that gelation does not occur before some of the carrageenan molecules have been converted to the double helix and the gel melts before all the double helices break up (graphs 2.1-2.3 and Table 2.1).

The ability of a mixture of two non-gelling systems to set up a firm gel indicates some interaction between the components and the evidence cited in this section points indirectly to binding of unlike chains.

5. Binding of Galactomannan Chains to the Carrageenan Double Helix

a) Involvement of Some Ordered Carrageenan Conformation

The presence of the carrageenan double helix is established in Section 3 but it remains to show its involvement with galactomannan in junction zones.

Synergistic gels set and melt fairly sharply as expected if the formation and break down of the junction zone is a co-operative process. This sharp melting and setting of gels has been taken to be a characteristic of gels formed by association of lengths of polymer chain held together by ordered non-covalent links. [107, 51] Most polysaccharide gelling systems show ordering of chain conformation in the junction zone as this permits repeating patterns of favourable bonding to be set up (eg. six hydrogen bonds in every repeat of the carrageenan double helix).[62]

b) Gel Point in Relation to the O.R. Transition

Examination of graphs 2.1-2.3 reveals that under no circumstances does gelation occur before O.R. indicates the conversion of some of the carrageenan molecules to the double helix. The position of melting and setting of the gels on the O.R. transition shows that conversion to the helical form is very small compared to gelling carrageenan systems (graph 1.1). In fact this level of conversion to helix never causes gel formation in carrageenan systems even in 1% KCl (graph 1.6). This indicates both the involvement of the carrageenan double helix and some contribution to the network from the galactomannan.

c) The Nature of the Non-Galactomannan Component

The synergistic activity of carrageenan, furcellaran[81a] and agarose[81] follows the trend shown in table 1.2 with agarose displaying very strong synergistic behaviour which decreases along the table to iota-carrageenan. Both undegraded iota-carrageenan and a non-gelling segmented product prepared by acid hydrolysis appear to have no synergistic activity with galactomannans as judged by gel formation.

Consideration of the distribution of the sulphate groups on the surface of the iota-carrageenan double helix (Fig. 1.5) shows that this structure bristles with bulky sulphate groups. It is possible that this will hamper the formation of long non-covalently bonded junctions with other polysaccharide molecules. Kappa-carrageenan, with no 2-sulphation of the anhydride residue, is similar to iota but with every second sulphate on each chain replaced by a hydroxyl group. Examination of the model (Fig.1.5) reveals that entire faces of the helix surface are sulphate free in the case of kappa-carrageenan. Kappa-furcellaran has about half the sulphate groups of Kappa-carrageenan and agarose none at all.

If the double helix is involved in synergistic junctions this factor would account for the variation in synergistic activity. This could be true, however, of any ordered carrageenan conformation.

An alternative explanation rests in the relation of the hydrophilic tendencies of the molecules to their degree of sulphation. Thus iota-carrageenan may not form synergistic junctions on account of the favourable solvation energy of isolated chains.

d) Stabilisation of the Double Helix by Locust Bean Gum

Although the O.R. characteristics of the helix-coil transition are largely retained, inspection of graphs 2.1-2.3 reveals that the transition is not completely unperturbed by addition of locust bean gum. This is demonstrated in graph 2.13 where the points at which the O.R. transition for a 2% segmented kappa-carrageenan solution is judged to commence and end are plotted against the concentration of galactomannan. The effect is greater for the end of the transition and appears to reach a point where the carrageenan is saturated with galactomannan at a carrageenan:locust bean gum ratio of about 2:1.

Graph 2.7, where the same points are plotted against the concentration of carrageenan, for a fixed galactomannan concentration (1% w/v) and in the absence of galactomannan, shows once more the optimum 2:1 ratio and the saturation of locust bean gum by carrageenan.

This can be rationalised if it is assumed that the locust bean gum allows the double helix to form more easily by binding to the transition state. This is analogous to the mechanism discussed in Chapter I for the nucleation of one carrageenan domain by another. The increased stability of the system on melting could also arise from the extra binding energy provided by the galactomannan-carrageenan junction.

The changes observed in the hysteresis patterns (graphs 1.1-1.3) reflect, as for the addition of KCl to a kappa-carrageenan system, the different changes in the stability of the transition state and the final product.

All this evidence suggests junction formation between the carrageenan double helix and regions of the galactomannan chains.

6. The Involvement of Unsubstituted Mannan Regions of the Galactomannan

As pointed out earlier the distribution of galactose residues along the mannan backbone of galactomannans is not random. The tendency for galactose stubs to occur in blocks must mean that some regions of the mannan chain of locust bean gum must be free from substitution, whereas fenugreek gum can have almost no such regions as there is one galactose residue for almost every mannose residue. To a rough approximation the ratio of galactose to mannose of a polymer will serve as a guide to the occurrence of chain regions free from galactose.

The synergistic activity (judged from gel formation with various carrageenans) can be represented as on Table 2.2. The ability of a galactomannan to gel non-gelling carrageenan solutions obviously depends on the Man/Gal ratio of that polymer and hence on the unsubstituted regions.

Further evidence has come from the behaviour of various modifications of locust bean gum.

Locust bean gum with a high Man/Gal ratio was prepared as described in the experimental section. It was found that this material had increased synergistic gel forming properties compared with the original. A mixture of 2% segmented carrageenan with 1% modified locust bean gum (graph 2.14) showed similar O.R. behaviour to mixtures containing normal locust bean gum (graph 2.3) but the transition is sharper and the hysteresis is more marked indicating greater interaction.

An attempt has been made[109] to prepare a locust bean gum of high Man/Gal ratio by use of an α-galactosidase enzyme. This experiment was partially successful in that the Man/Gal ratio was increased. However the enzyme was contaminated with β-mannanase and the molecular weight of the galactomannan was reduced, so that the product had no synergistic activity. In fact it had some interesting anti-synergistic effects in preventing the gelation of some agarose and carrageenan systems which gel in the absence of this material. If this product still attaches to the agarose or carrageenan helix, but is not long enough to take part in two such junctions, no contribution to the network will be achieved. In systems where the agarose or carrageenan chains are normally dependent on aggregate junctions for gelation this material could bind to the forming network and block formation of these aggregate

junctions, thus preventing gelation. This implies, however, that the galactomannan chain hinders aggregation whereas some observations suggest the opposite (see section 7(d))

7. The Nature of the Junction Zones

It has been shown that the carrageenan component is present in synergistic junction zones in the double helix form. The galactomannan chain is also likely to adopt an ordered conformation in the junction zone for the reasons outlined in section 5(a).

Computer model building studies indicate that the only ordered conformation that a β-1,4-mannan chain can adopt is some type of extended ribbon-like helix[110]. This is borne out by X-ray studies on guar gum[111] and other polysaccharides with a β-1,4-mannan backbone.[112-114] Therefore it is likely that the galactomannan occurs in the junction zone in such a conformation. Fig. 2.4 shows schematically how this junction zone could be formed between ordered regions of each polymer chain.

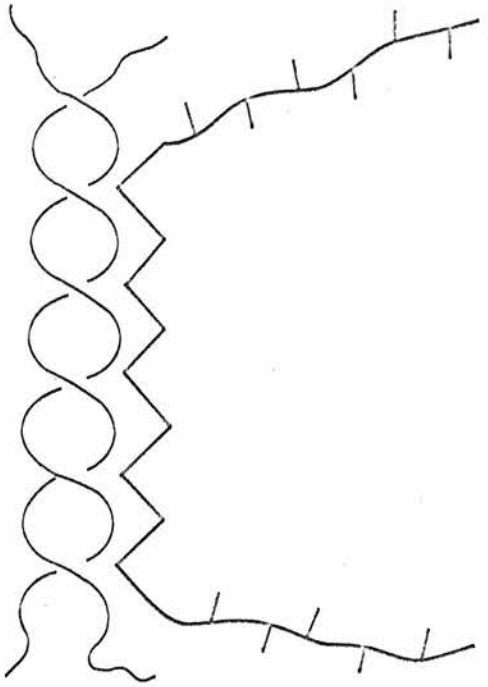


Fig. 2.4. Proposed model for a synergistic junction zone.

This leaves several important questions unanswered.

(a) What is the minimum length of the junction?

(b) What is the geometry of the association and what are the stabilising forces involved?

(c) What is the "stoichiometry" of the association? How many binding sites for galactomannan are there on each carrageenan molecule and vice versa?

(d) How many chain segments of each polymer are involved in a junction?

(a) An attempt has been made to find an answer to this question for the agarose-galactomannan system by controlled periodate oxidation of locust bean gum chains.[115] Products were isolated after oxidation had been allowed for 2,4,10,17,20 and 24 minutes at 3-4°C. The reaction was then stopped by addition of excess of ethylene glycol and the products were reduced with borohydride. All residues of a galactomannan are susceptible to periodate oxidation but we are interested in the unsubstituted mannan regions. Here some of the mannose units are converted to open chain residues but the chain is not degraded. These residues act in an analogous fashion to the kinking residues of carrageenan. The chains were subsequently degraded by mild hydrolysis. Analysis of the degradation products showed that more than 30 mannose residues are probably involved in a junction. If these products are subjected to mild hydrolysis short segments are obtained which display anti-synergistic behaviour similar to that shown by the enzymically degraded products.

(b) All that can be said about the geometry of the junction zone is implicit in the ordered conformations adopted by the two components. The effect of sulphation on the non-galactomannan component may give some idea of the necessary areas of close contact.

(c) It must be assumed, since galactomannans cause segmented carrageenan to gel that each galactomannan molecule has at least two binding sites. The antisynergistic behaviour of the degraded galactomannan chains is probably brought about by the existence of only one binding site on each molecule.

(d) The number of chains in each junction zone is not immediately obvious. It is generally reported that the use of galactomannans in industrial gels clarifies the gel, makes it more elastic and reduces syneresis. Thus all the indications suggest that chain aggregation is reduced, probably on account of physical blocking by bound galactomannan chains.

Industrial gels of agarose are of relatively high concentration and kappa-carrageenan gels are usually formed in strong KCl. Thus these gels have a very turbid appearance indicating extensive chain

aggregation. Synergistic gels studied in this thesis were formed in the absence of added salt and turbidity appeared to increase on addition of galactomannan. The same effect was observed for synergistic gels formed from dilute agarose systems.[81]

The independence of the position of the O.R. transition of carrageenan - locust bean gum solutions (graph 2.7 and 2.13) on polymer concentration (at higher polymer concentrations) indicates that the enthalpy change for the transition is high (Chapter I). This high enthalpy could indicate the co-operative formation of fairly large aggregates.

The increased turbidity of these systems is not wholly inconsistent with industrial observations as the degree of chain aggregation could be significantly reduced without preventing the formation of aggregate junctions. There is, however, no evidence to suggest that simple junctions of the type shown in Fig. 2.4 do not occur.

8. Quaternary Structure of a Polysaccharide

The synergistic behaviour of the carrageenan-galactomannan system can be described as an example of polysaccharide quaternary structure

by drawing a parallel with protein structure as follows. Proteins and polysaccharides are linear polymers built up from amino acid and monosaccharide residues respectively. The nature and sequence of these residues is known as the primary structure of the polymer. The secondary structure of a macromolecule describes the ordered conformation adopted by the polymer chain. In proteins the α-helix is the classic secondary structure; in polysaccharides secondary structure is represented by the shape taken up by one chain in the carrageenan double helix or in the xylan triple helix. The term tertiary structure is used in protein systems to describe such molecular organisation as the folding of the amino acid chain in an enzyme subunit or the relation of the chains to each other in the collagen triple helix. The arrangement of the polymer chains in the carrageenan double helix or the xylan triple helix are also examples of macromolecular tertiary structure. The co-operative binding of the ordered structures of two unlike polysaccharides in synergistic systems represents the first example of polysaccharide quaternary structures. This level of structural organisation is familiar in proteins in the subunit structure of most enzymes.

9. Ligand Induced Changes in Polysaccharide Conformation

Ligand induced conformation changes in proteins are discussed in the introduction to this thesis. Such changes can be initiated by a variety of ligands including other protein molecules (interaction between protein subunits in Koshland's explanation of allosterism).

The carrageenan-galactomannan system exhibits ligand induced changes of polysaccharide conformation. Firstly the coil to double helix transition of the carrageenan occurs at a higher temperature in the presence of galactomannan than in its absence (graph 1.7 and 1.13). A more dramatic effect is produced on the conformation of the galactomannan chain by the presence of the ordered carrageenan conformation. The ribbon-like conformation adopted by the mannan segments of the chain can only be achieved in solution when they co-exist with carrageenan in synergistic junction zones.

10. Other Synergistic Polysaccharide Systems

a) Agarose and Galactomannans[116, 81]

Agarose displays similar synergistic gelling activity, but the phenomenon is more marked than for carrageenans. This fits into the

trend from iota to Kappa to furcellaran. The most interesting feature of this system is the effect that galactomannan addition has on O.R. behaviour. Agarose solutions alone show a negative change in O.R. when cooled and a very large hysteresis loop is obtained on reheating (Fig. 2.5(a)). When galactomannan is added the sign of the shift is changed and the curious butterfly shaped curve shown on Fig. 2.5(b) is obtained. This reversal in the sign of the shift is attributed to an overwhelming positive contribution from the change in the chain conformation brought about by rotation about the bond angles between mannose residues when the chain takes up the ribbon-like conformation. On the reheating curve the cross over is assumed to represent melting off of the galactomannan and the system then shows normal agarose behaviour.

Further support of the synergistic model comes from examination of the liquid released from an agarose-galactomannan gel by freeze-thaw treatment. This is found to contain galactomannan of low Man/Gal ratio indicating that material with a high Man/Gal ratio is being bound.

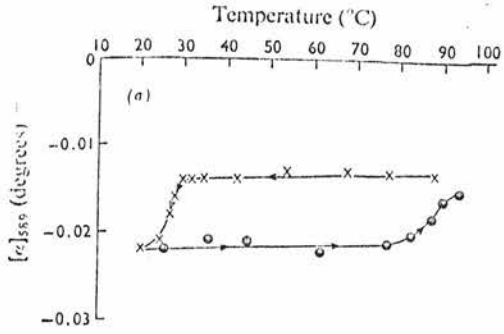


Fig. 2.5(a). Variation of optical rotation with temperature for a solution of agarose (0.05%).

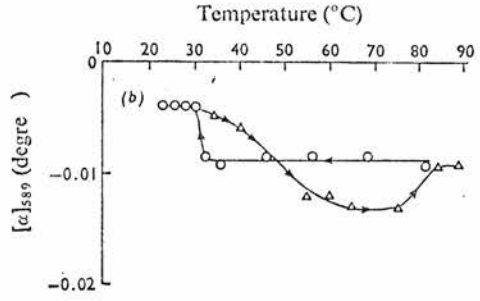


Fig. 2.5(b). Variation of optical rotation with temperature for a solution of an agarose (0.05%) - galactomannan (0.1%) mixture.

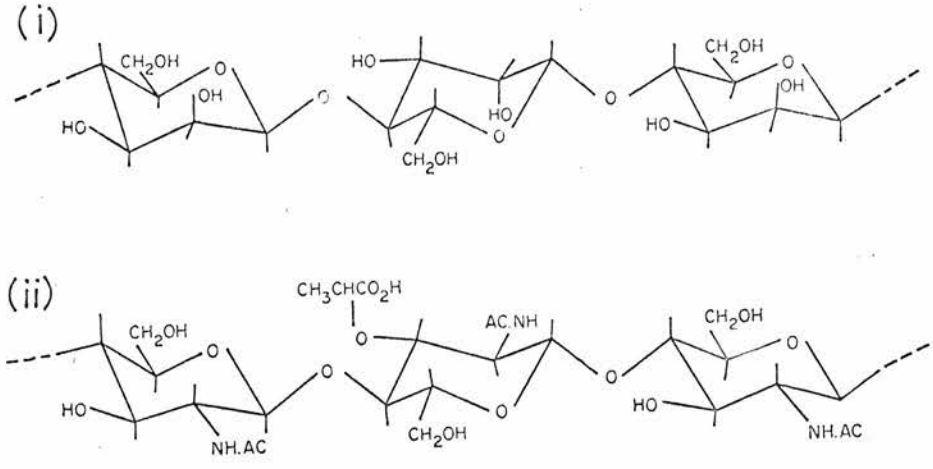


Fig. 2.6. Comparison of the backbone structures of mannan (i) and peptidoglycan (ii).

The extracellular polysaccharide from Xanthamonas campestris does not gel alone but forms a firm gel with high Man/Gal galactomannans.[3] As in the agarose case a positive O.R. contribution from the galactomannan can be observed.

On the basis of minimal gelling concentrations, this polysaccharide and kappa-carrageenan appear to have roughly equal synergistic activity. It might thus be expected that, if the galactomannan conformation adopted is the same, the O.R. contribution due to the galactomannan would also be observed in the carrageenan case. The specific O.R. change shown with the bacterial polysaccharide is of the order of $+4^{\circ}$. Because all carrageenan measurements had to be carried out in a 0.1 dm cell (because of turbidity) this would represent an actual O.R. change of 0.004 for systems with 1% w/v galactomannan. Since accuracy of measuring O.R. is $\pm$ 0.002 such changes could be present in the carrageenan system, especially for the low Man/Gal ratio material (graph 2.14).

11. A Model for the Cohesion of Cell Wall Polysaccharides

Certain other polysaccharides show synergistic gel formation

both with agarose and kappa-carrageenan. Gels were formed at room temperature by a solution 2% with respect to kappa-carrageenan and 1% with respect to either carboxymethyl cellulose (β-1,4-glucan backbone) or sapote gum (β-1,4-xylan backbone) or tamarind seed mucilage (β-1,4-glucan backbone). The heavily substituted xylan from corm sacs of Watsonia pyrimidata did not form a synergistic gel under these conditions. The O.R. versus temperature behaviour of a C.M.C - carrageenan system is shown in graphs 2.5 and 2.6. The common structural feature of the molecules which form synergistic gels with carrageenan is a relatively unsubstituted β-1,4-mannan, glucan or xylan backbone. Glucose and mannose are C(2) epimers and xylose is identical to glucose apart from the equitorial substituent on C(5). These molecules are very similar in structure to skeletal polysaccharides such as the hemicelluloses, cellulose (fig. 0.5) and peptidoglycan (fig. 2.6). This suggests that the synergism of agarose and carrageenan may reflect associations of these polymers with the polysaccharide chains of the skeletal materials with which they co-exist.

12. Limitations of Methylene Blue as a Carrageenan Conformation Probe

It has been known for some time that carrageenan interacts with certain cationic stains and can be precipitated by them.[117]

Changes in the absorption spectrum of such dyes can be brought about by the addition of many polyanions including polysaccharides.[118-120] This phenomenon, known as metachromasy, has been shown for carrageenan systems.[121] Williamson[62] has shown, by C.D., optical rotary dispersion (O.R.D.) and U.V. spectroscopy measurements, that there is scope for the use of this interaction as a conformational probe in carrageenan systems.

In the present study it was hoped that the appearance of the CD spectrum and the change in shape of the U.V. absorption on cooling a kappa-carrageenan/methylene blue solution (with and without galactomannan) could be correlated with the O.R. behaviour of kappa-carrageenan and kappa-carrageenan-galactomannan systems and thus serve as further evidence for the ligand-induced conformation change (section 9).

In graphs 2.9 and 2.10 the appearance of C.D. bands and the U.V. spectrum change is shown for a solution of kappa-carrageenan segments (2%) and methylene blue (9.8×10^{-4}%). A solution of methylene blue (9.8×10^{-4}%) showed little change on heating from 20°C to 70°C. The height of one of the U.V. peaks (668 nm) and the C.D. peak at 555 nm are plotted against temperature in graphs 2.15 and 2.16 respectively.

Comparison of graphs 2.15 and 2.16 with graph 1.3 reveals that the appearance and disappearance of these C.D. and U.V. peaks do not follow the O.R. transition: both heating and cooling branches of these curves occur at higher temperature and hysteresis is increased.

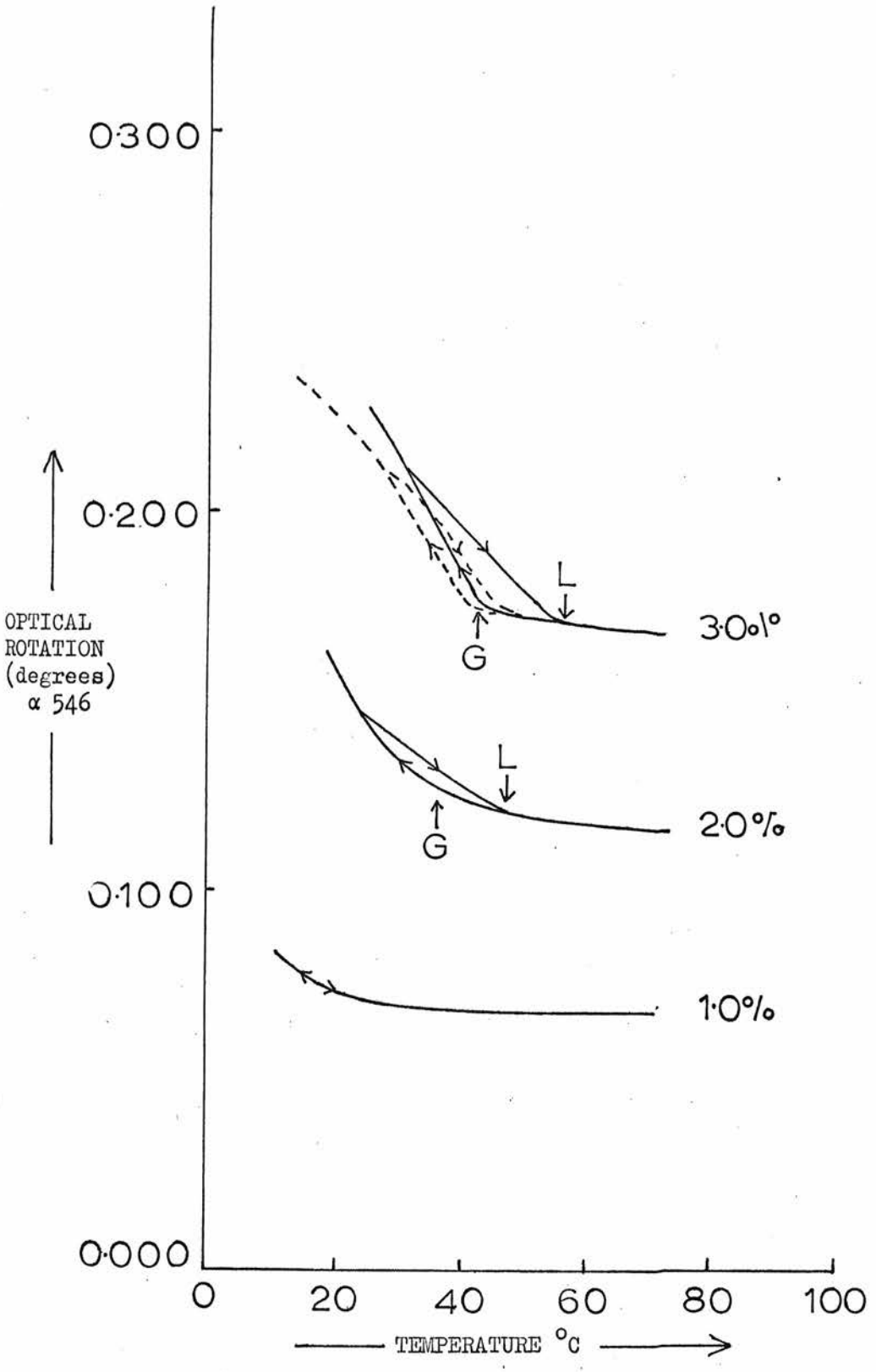
The commonly accepted theory of metachromasy[122] proposes that, in the presence of suitable linear polyanions, the dye molecules bound to adjacent sites on the polymer interact with each other and delocalisation of the π electrons of the aromatic rings leads to the observed spectral changes. C.D. spectra are induced as the chromophore is bound to an asymmetric environment, the spectra being greatly enhanced by dye stacking. In mucopolysaccharide-dye systems, metachromasy is dispelled at relatively low polymer site/dye (P/D) ratios[123,124] but the methylene blue spectral changes occur in carrageenan systems at a P/D ratio of at least 2000:1. O.R. measurements on carrageenan systems containing methylene blue ($9.8 \times 10^{-4}\%$) are identical to those obtained in the absence of dye. This would be expected if the spectral changes of the dye reflect the conformational state of only a small fraction of the carrageenan molecules. Such behaviour implies that the stability derived from dye stacking (evident from self-aggregation in cold concentrated dye solutions[125]) is sufficient to cause co-operative dye binding to the double helix, a

polymer conformation with suitably arranged sites. An attempt to influence the O.R. behaviour by having a P/D ratio of 100:1 resulted in precipitation of the carrageenan/dye complex.

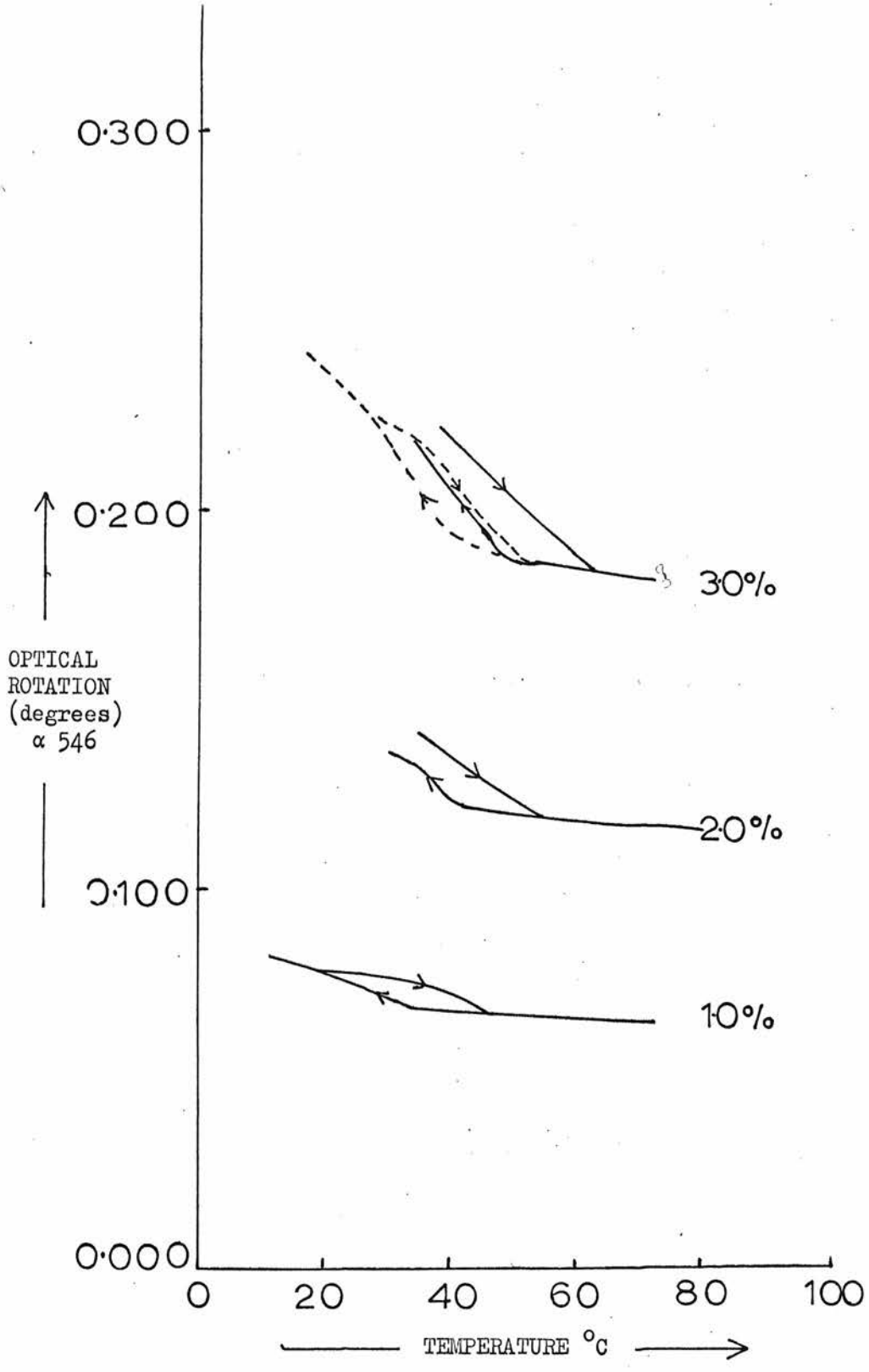
It is possible that the methylene blue reflects an unperturbed change of a few carrageenan molecules but it would appear that the energy provided by the interaction of the aromatic dyes is responsible (cf. section 9) for "ligand induction" of the conformation change of a few molecules. Thus these "reporter molecules" do show the conformational state of the carrageenan in the complex but on account of the tendency of the dye to drive the coil to double helix transition, care must be taken in using such evidence as an indication of polymer conformation in the absence of dye. These reservations, of course, also apply to mucopolysaccharide-dye complexes.[124]

It would be useful to find some chromophore which does not "stack" but which binds randomly to these polymers, giving a C.D. spectrum when bound on account of its assymetric environment, but which can be used in small enough quantities not to cause an added salt effect.

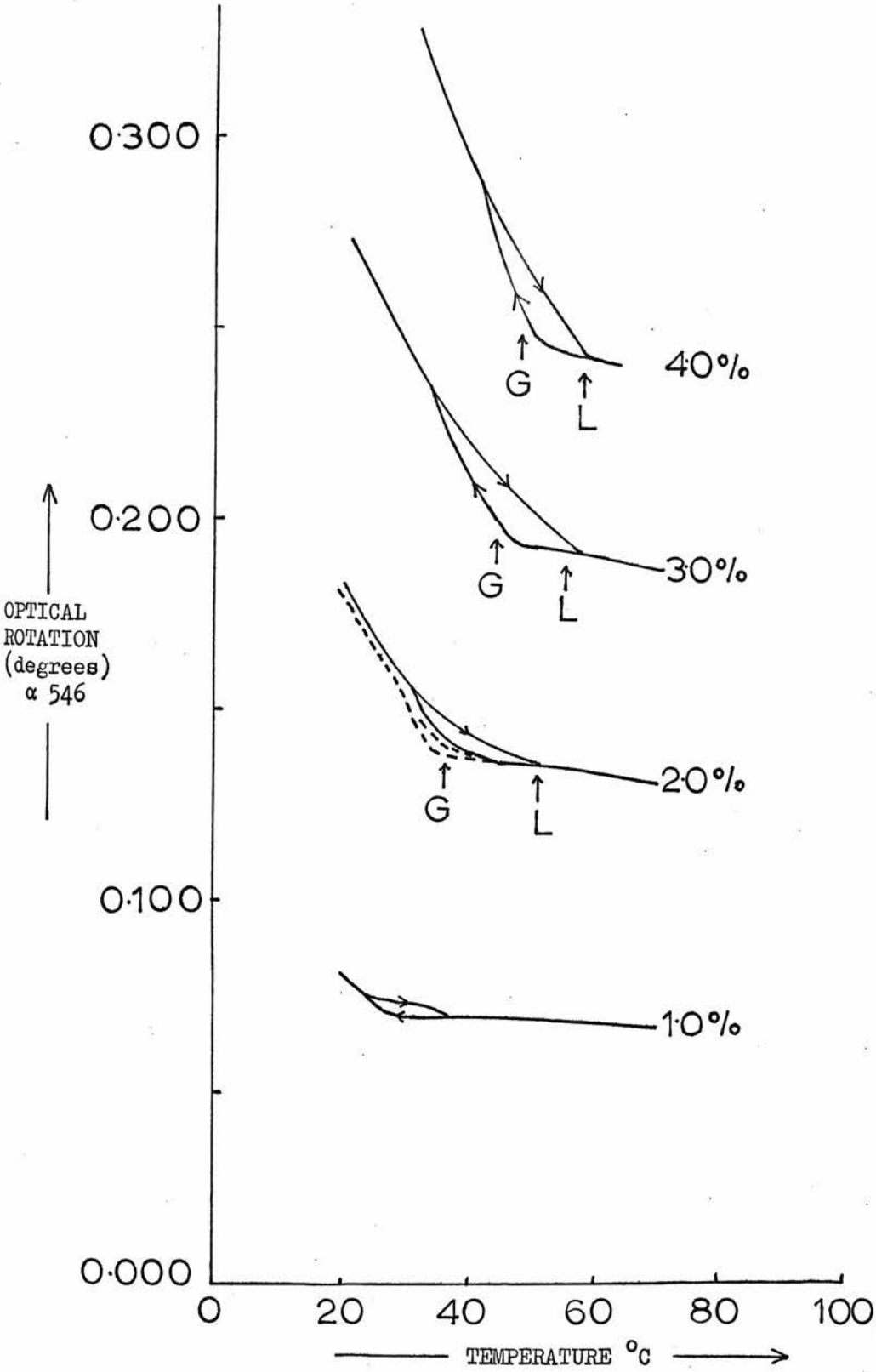
D. GRAPHS AND TABLES



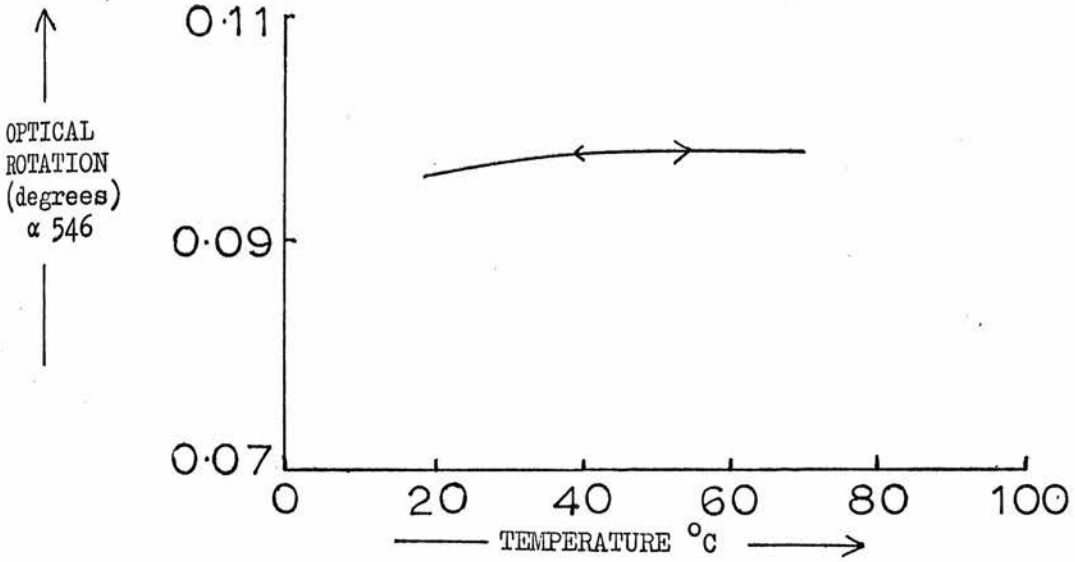
Graph 2.1. Variation in optical rotation on heating and cooling aqueous solutions of kappa-carrageenan (Chondrus crispus) - locust bean gum (1%) mixtures. Gel points (G) and liquefaction points (L) are shown. Melting and setting points of synergistic gels occur over a relatively large temperature change.



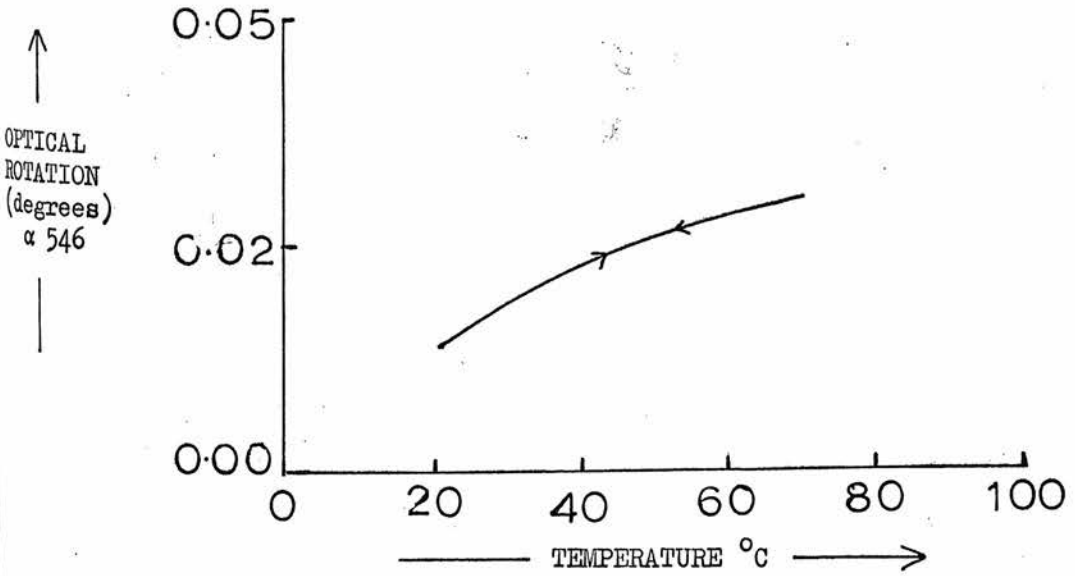
Graph 2.2. Variation in optical rotation on heating and cooling aqueous solutions of kappa-carrageenan (Eucheuma cottonii) - locust bean gum (1%) mixtures.



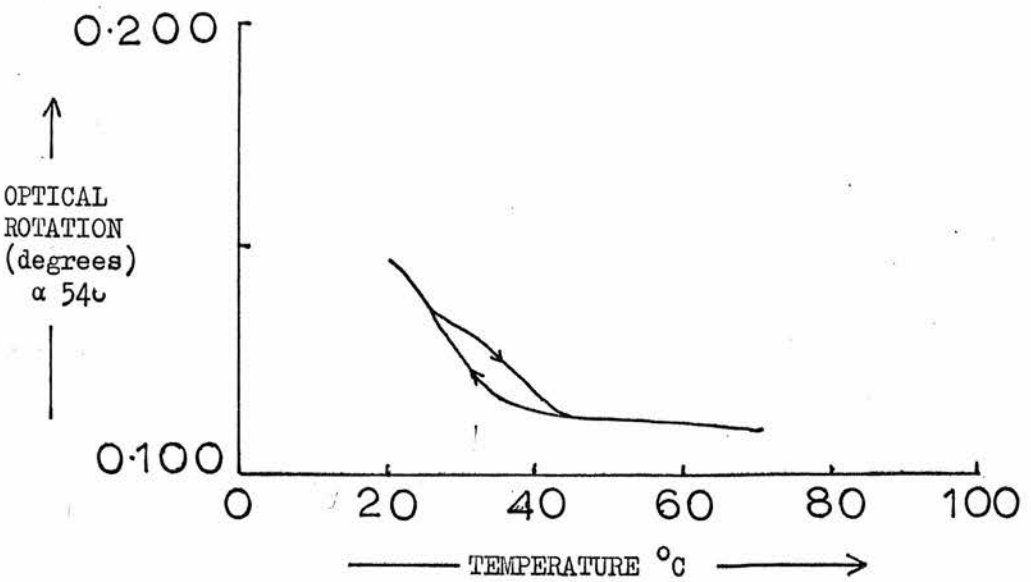
Graph 2.3. Variation in optical rotation on heating and cooling aqueous solutions of segmented kappa-carrageenan - locust bean gum (1%) mixtures. Gel points (G) and liquefaction points (L) are shown.



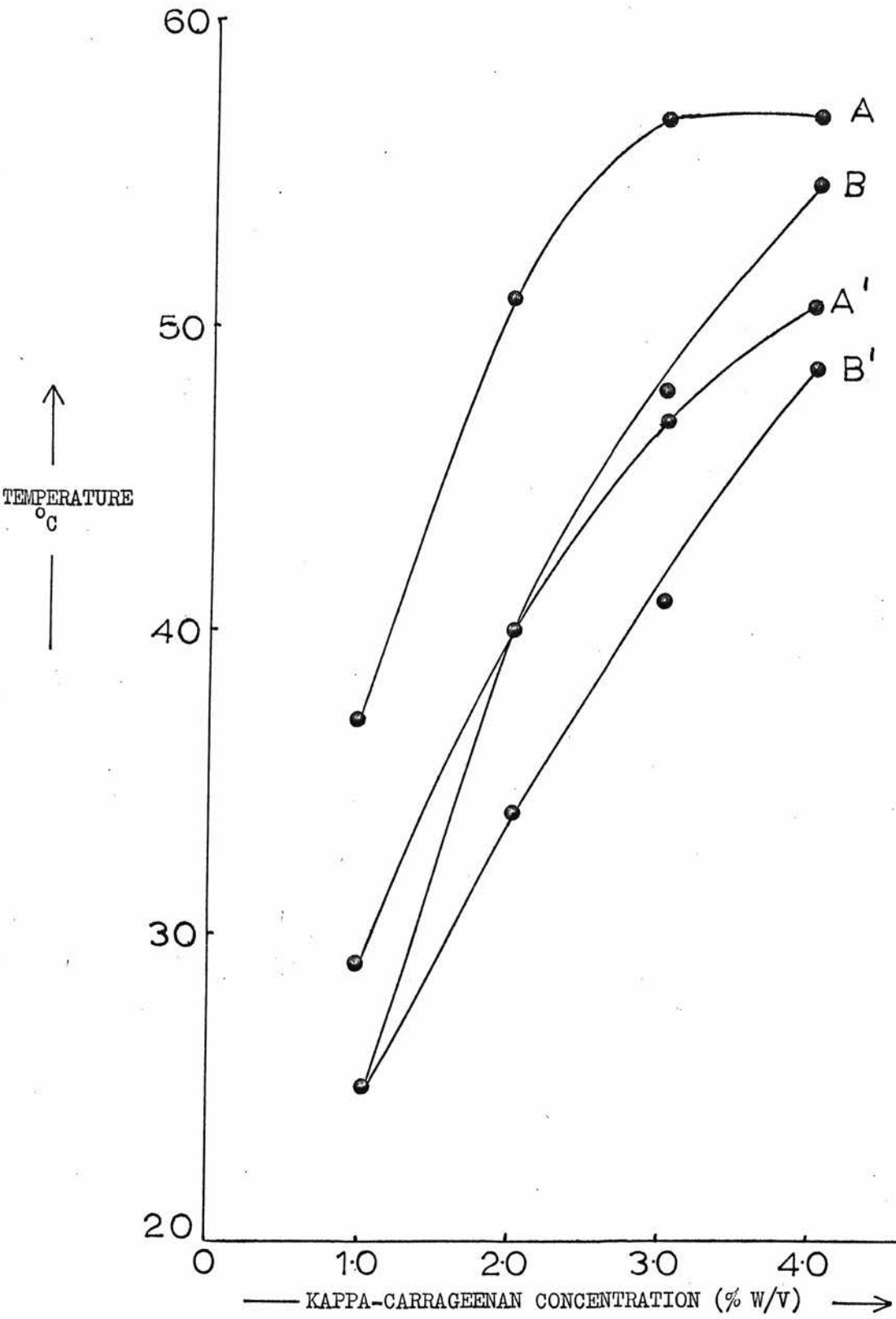
Graph 2.4. Variation in optical rotation on heating and cooling an aqueous solution of locust bean gum (1%). Measurements were made in a 1 dm cell.



Graph 2.5. Variation in optical rotation on heating and cooling an aqueous solution of carboxymethyl cellulose (0.6%). Measurements were made in a 1 dm cell.

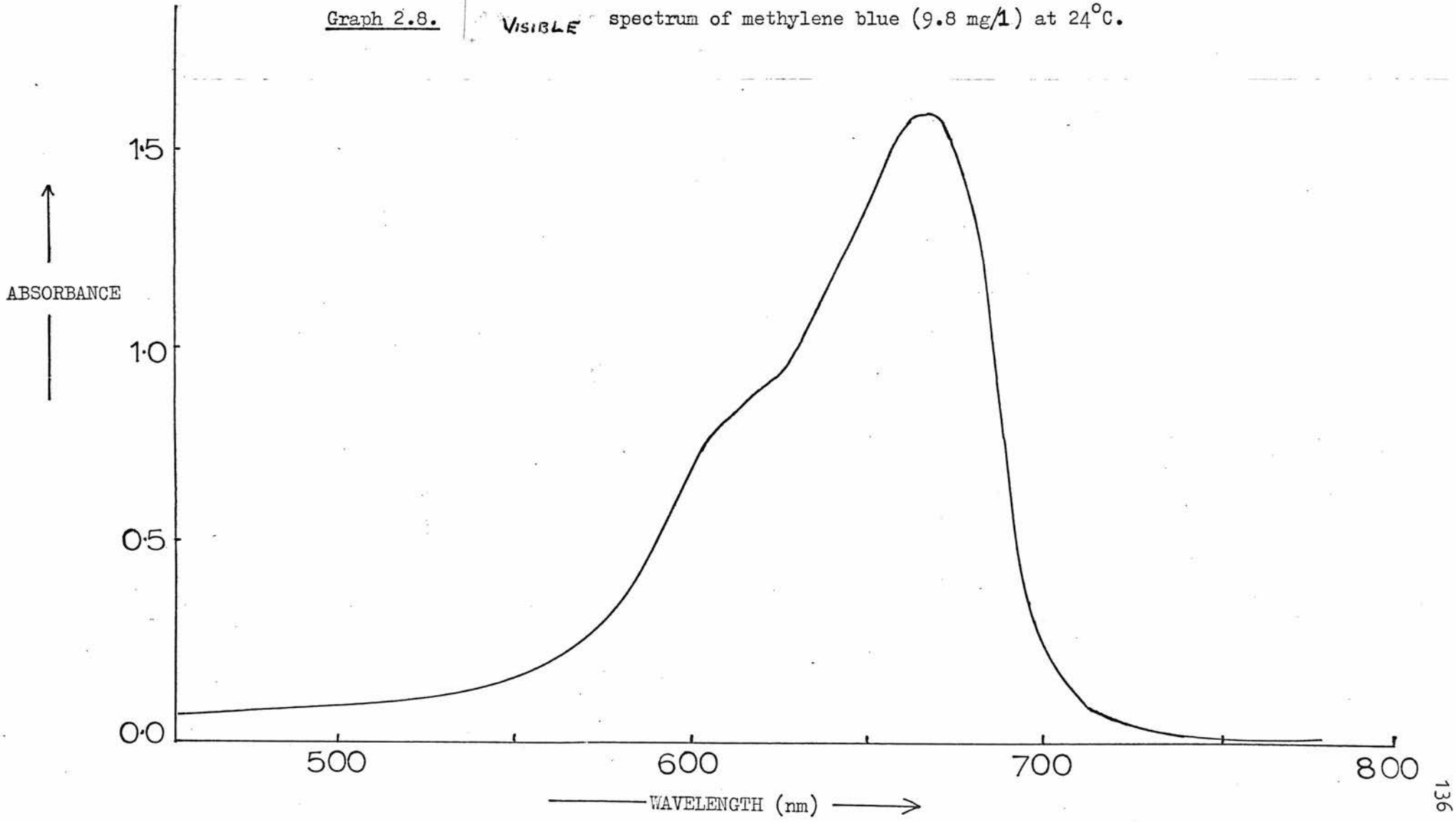


Graph 2.6. Variation in optical rotation on heating and cooling an aqueous solution of kappa-carrageenan (2%) and carboxymethyl cellulose (0.5%).

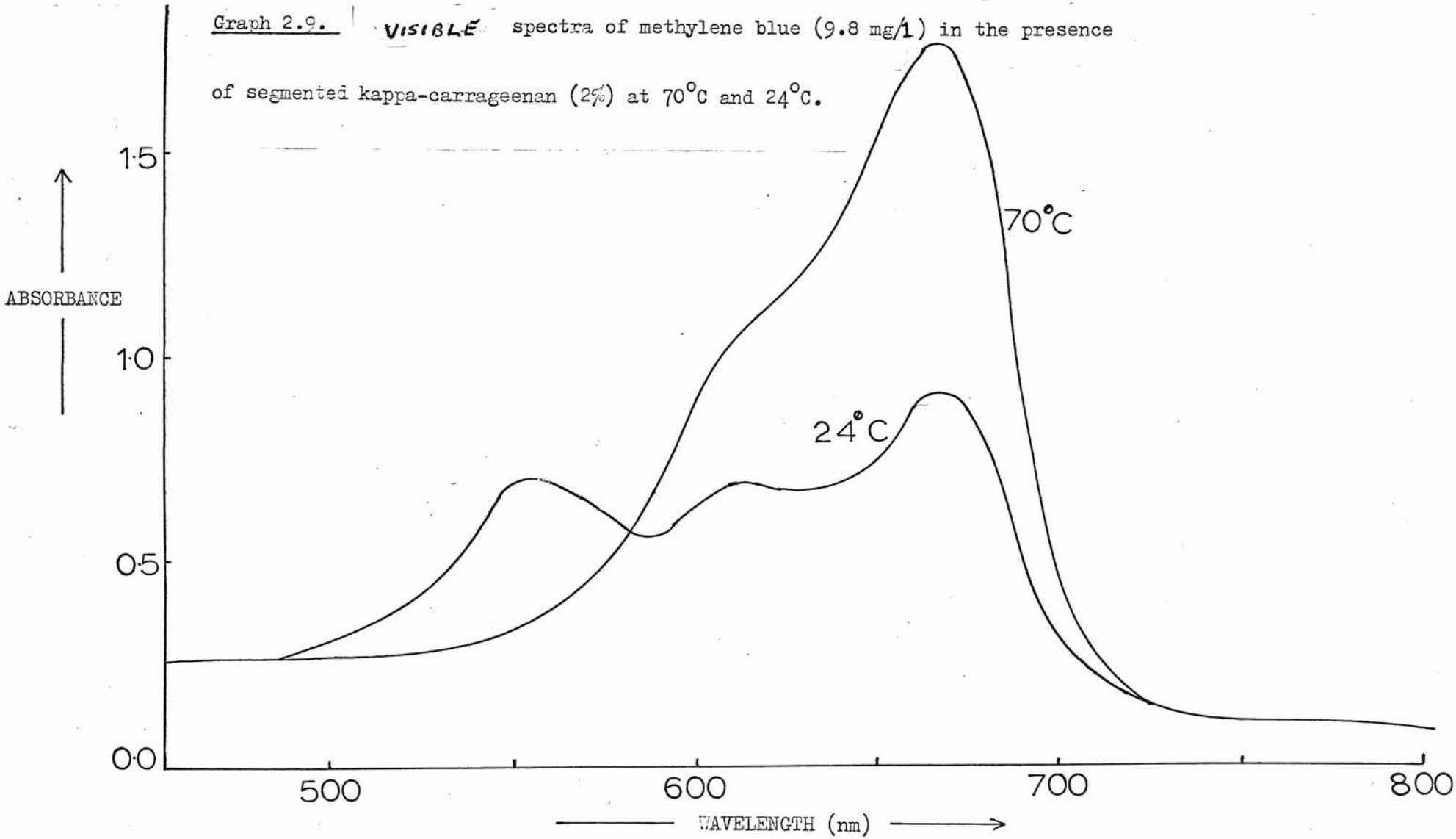


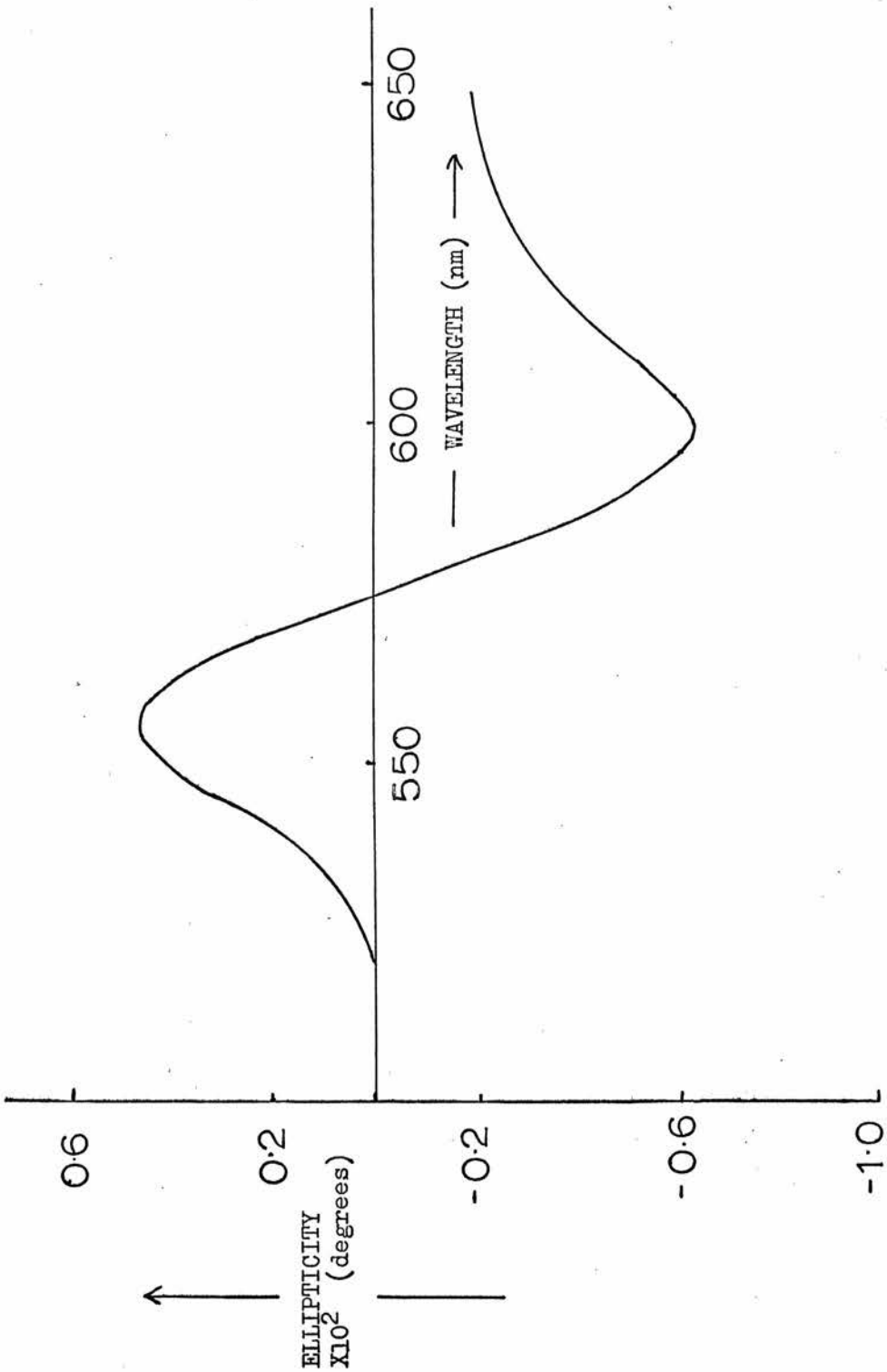
raph 2.7. Variation in the temperature of the end (curve A) and the commencement curve A') of the optical rotation transition of segmented kappa-carrageenan and locust ean gum (1%) solutions. The same points are shown for kappa alone (curves B and B').

Graph 2.8. VISIBLE spectrum of methylene blue (9.8 mg/1) at 24°C.



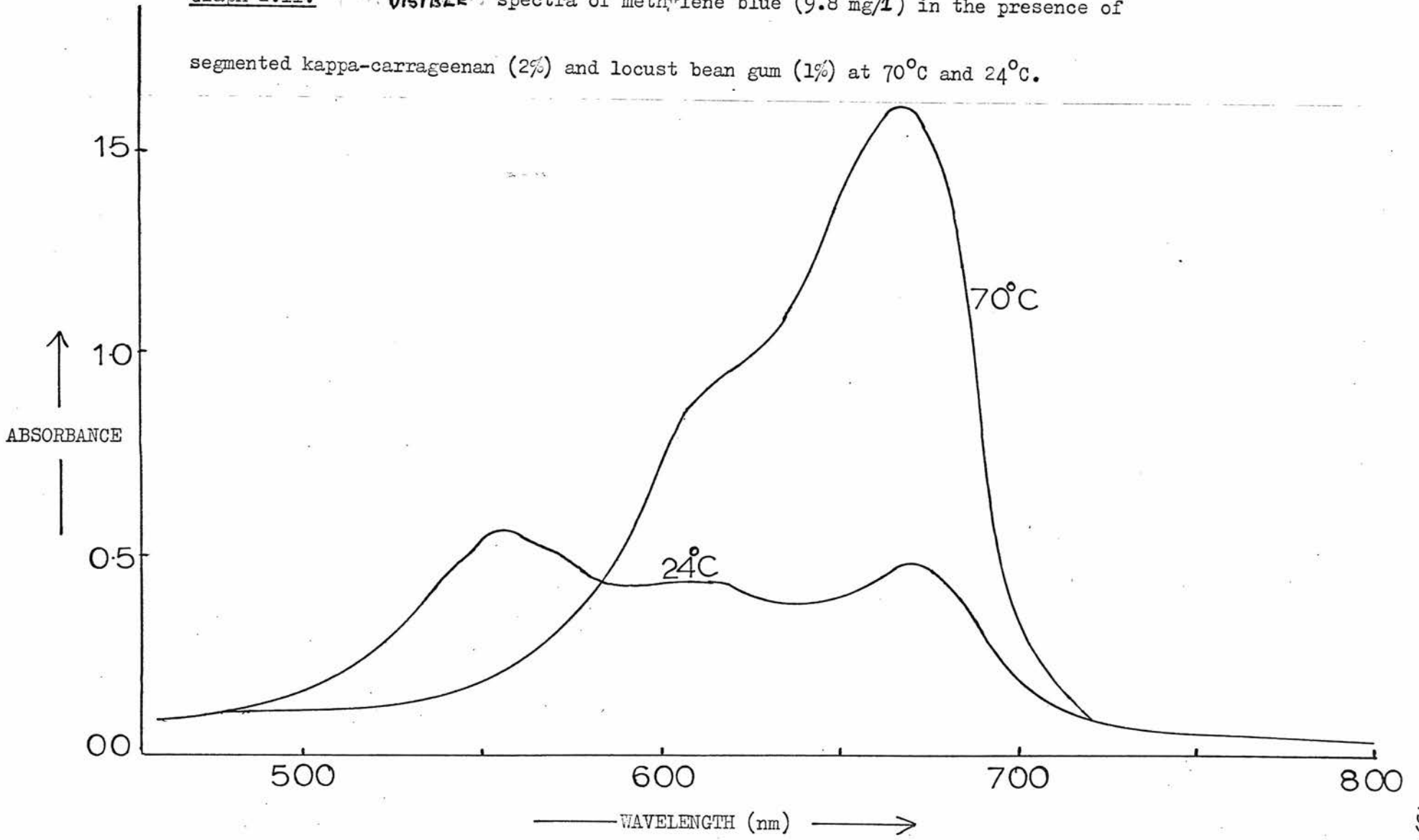
Graph 2.9. VISIBLE spectra of methylene blue (9.8 mg/l) in the presence of segmented kappa-carrageenan (2%) at 70°C and 24°C.

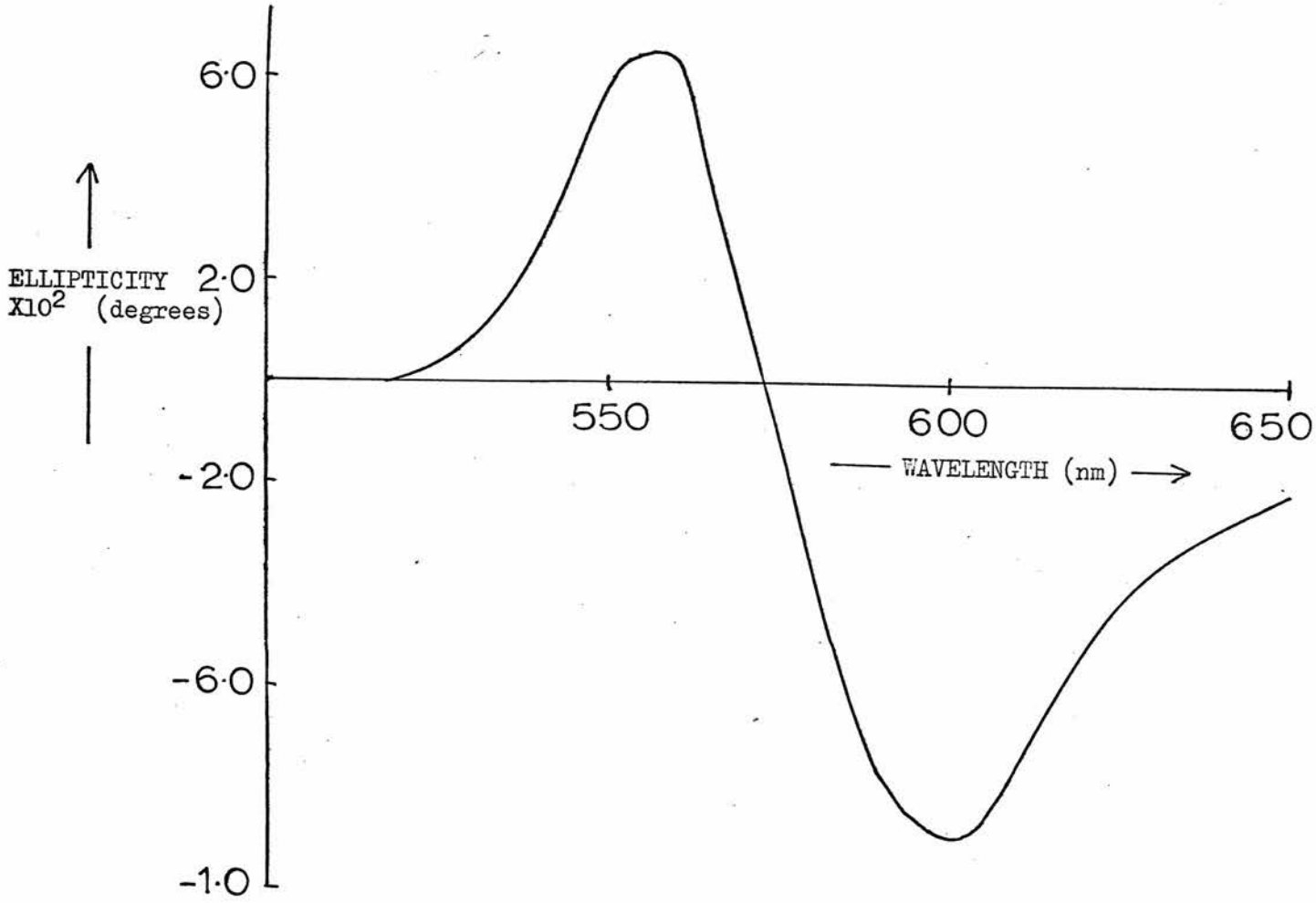




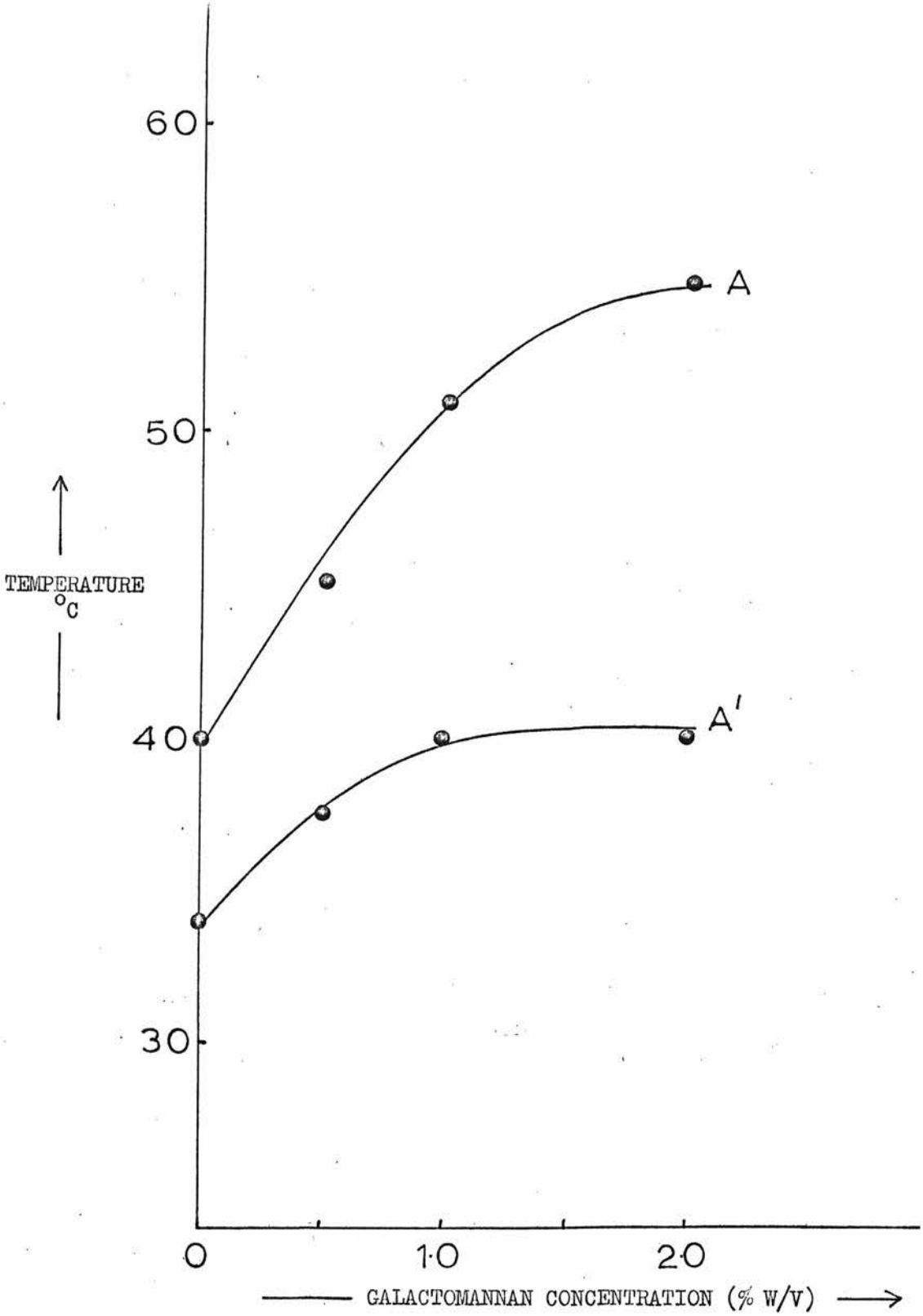
Graph 2.10. Circular dichroism spectrum of methylene blue (9.8 mg/l) in the presence of segmented kappa-carrageenan (2%) at 24°c.

Graph 2.11. VISIBLE spectra of methylene blue (9.8 mg/l) in the presence of segmented kappa-carrageenan (2%) and locust bean gum (1%) at 70°C and 24°C.

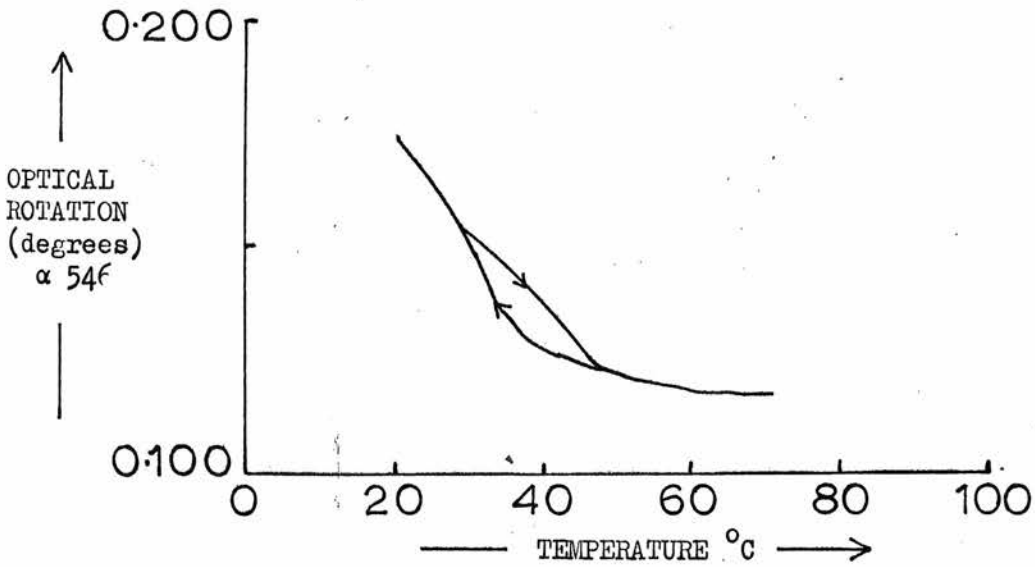




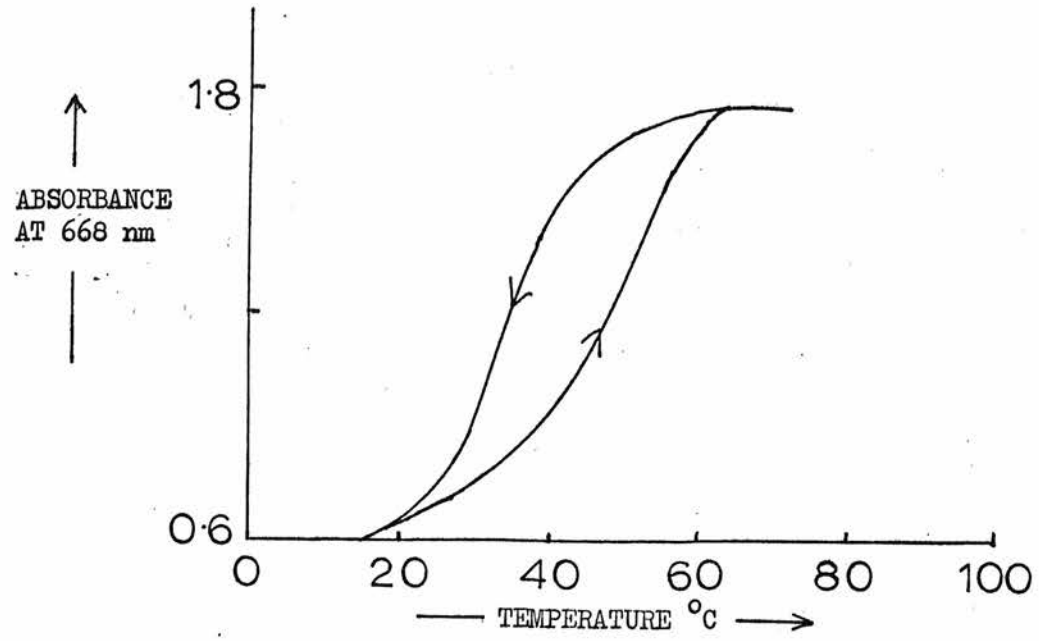
Graph 2.12. Circular dichroism of methylene blue (9.8 mg/l) in the presence of segmented kappa-carrageenan (2%) and locust bean gum (1%) at 24°C.



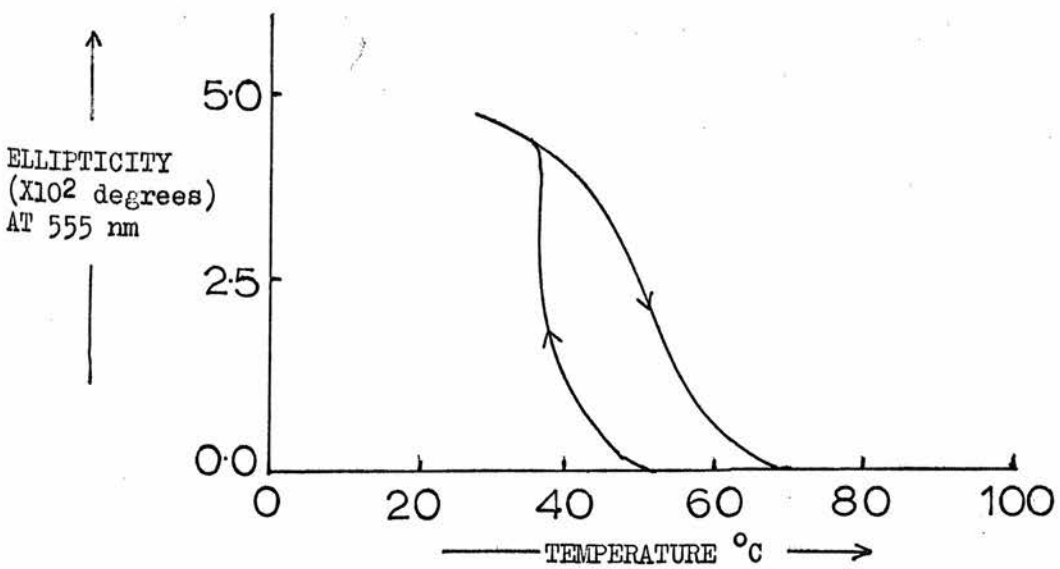
Graph 2.13. Variation in the temperature of the end (curve A) and the commencement (curve A') of the optical rotation transition of a mixture of segmented kappa-carrageenan (2%) and locust bean gum with concentration of locust bean gum.



Graph 2.14. Variation in optical rotation on heating and cooling an aqueous solution of a mixture of segmented kappa-carrageenan (2%) and locust bean gum of a high Man/Gal ratio (1%).



Graph 2.15. Variation in the absorbance at 668 nm when heating and cooling a methylene blue solution (9.8 mg/l) in the presence of segmented kappa-carrageenan (2%).



Graph 2.16. Variation in the ellipticity at 555 nm when heating and cooling a methylene blue solution (9.8 mg/l) in the presence of segmented kappa-carrageenan (2%).

Table 2.1. Temperatures (^{o}C) of gelation (G) and liquefaction (L) of aqueous kappa-carrageenan - locust bean gum mixtures.

SAMPLE	KAPPA-CARRAGEENAN (% W/V)					
	2%		3%		4%	
	G	L	G	L	G	L
Chondrus crispus	36	47	42	56		
Segmented	36	51	44	55	48	58

Table 2.2. Synergistic activity of various galactomannans as judged by their ability to gel non-gelling concentrations of carrageenan-like polysaccharides.

Galactomannan	Man/Gal	Synergistic Activity
Locust bean gum	3.35	+++++
Tara gum	3.00	++++
Guar gum	1.56	++
Fenugreek gum	1.08	+

OPTICAL ROTATION BEHAVIOUR OF SOME POLYSACCHARIDES

A. Introduction

The measurement of optical rotation at a single wavelength has been extensively employed, as described in the preceeding chapters, to follow changes in chain conformation. Such optical rotation changes have been placed on a quantitative basis by assuming that the shape of the individual sugar rings remains constant [67]. By consideration of quantum mechanical approaches Kauzmann et al [126] have shown that optical rotation phenomena do not arise from a property of individual groups, such as the motion of electrons in an atom, but from the effect of interaction between groups on such motions. Thus optical rotation is not, as suggested by the van't Hoff superposition principle, a sum of contributions from isolated groups, but a sum of the interactions between them. This explains the limitation of Hudson's rules which are based on the van't Hoff superposition principle.

By consideration of dominant interactions Whiffen [127] and Brewster [128] have derived a set of empirical rules which predict the optical rotation of many compounds including monosaccharides.

To extend this treatment to polysaccharides and to relate chain conformation to optical rotation it is necessary to relate the dihedral angles ϕ and ψ (Fig. 3.1) to that contribution to the optical rotation which arises from interactions across the glycosidic bridge. Rees [67] introduced the term "linkage conformation" to denote a particular pair of

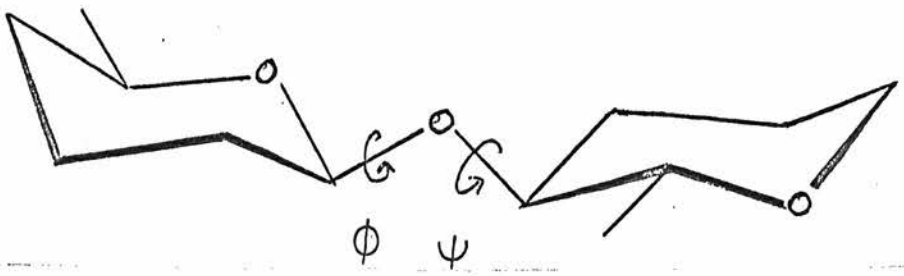


Fig. 3.1 The dihedral angles ϕ and ψ. A definition of the zero positions and the positive directions of $\Delta\phi$ and $\Delta\psi$ are defined in reference 67.

values for ϕ and ψ. He also defined the "linkage rotation" of a disaccharide. For a disaccharide with a reducing residue (R) and a non-reducing residue (N) the linkage rotation $[\Lambda]$ can be expressed as

$$[\Lambda] = [M_{NR}] - \left[[M_{MeN}] + [M_R] \right]$$

where $[M_{NR}]$ is the molecular rotation of the disaccharide, $[M_{MeN}]$ is the molecular rotation of the methylglycoside of N (with the same anomeric configuration as the disaccharide) and $[M_R]$ is the molecular rotation of the reducing sugar.

Using Brewster-type assumptions about dominant effects an expression was derived by consideration of interactions in chains of bonded atoms taken four at a time. The expression differs for α - and β - linked disaccharides and is

$$[\Lambda^{\beta}]_D = + 105 - 120 \ (\sin\Delta\phi + \sin\Delta\psi)$$

$$[\Lambda^{\alpha}]_D = - 105 - 120 \ (\sin\Delta\phi + \sin\Delta\psi)$$

The expression also changes for residues in the L configuration and for those not in the Reeves C1 conformation.

The validity of these expressions was confirmed by the agreement between observed and calculated optical rotations for di - and oligo - saccharides for which solution conformations could be predicted from X-ray and nmr data. Correlation with X-ray studies is obviously not possible when the crystal conformation is not retained in solution but analysis of the changes between crystal and solution can provide useful insights [67]. Further supporting evidence for the expression has come from comparison of measured optical rotations with values based on chain conformations which have been predicted by computer model building [110].

The expressions are not valid as they stand in the presence of certain chromophores, such as are found in acetamido sugars or uronic acids, due to the relative proximity of the Cotton effects of these chromophores to the sodium D line (589 nm).

In this chapter a series of carbohydrate polymers from a wide variety of sources has been surveyed in a search for sharp sigmoidal optical rotation

changes during heating and cooling. Although the presence of such changes is indicative of co-operative conformational changes their lack does not preclude the adoption of a specific chain geometry. For example in pectin systems[115], where the chains are known to form ordered microcrystallites, no significant changes are observed as ψ and ϕ in the random coil appear to oscillate about the values adopted in the fixed geometry of the microcrystallite.

B. Experimental

Materials

These are described in the "General Methods" section.

Measurement of Optical Rotation versus Change of Temperature

The preparation and clarification of laminaran solutions and the method of measurement of optical rotation against temperature is described in the "General Methods" section. These measurements were carried out in water, dimethyl sulphoxide (DMSO) and formamide for insoluble laminaran and in water and DMSO for soluble laminaran. The results are shown in graphs 3.1 and 3.2. The optical rotation - temperature behaviour of laminaribiose was also recorded (graph 3.3). The results were corrected for the change of refractive index and density which occurs on heating by multiplying by the conversion factor (K) [129] where

$$K = \frac{\eta_{25}^2 + 2}{\eta_T^2 + 2} \cdot \frac{\rho_{25}}{\rho_T}$$

η_{25} and ρ_{25} are the refractive index and density of the solvent at 25°C and η_T and ρ_T are the equivalent values at a temperature T.

The measurement of optical rotation against temperature for the other polysaccharide systems discussed in this chapter was carried out as described below. Polysaccharide solutions (Table 1) were made up by heating in a pressure cooker (122°C). The solutions were filtered through hot millipore filters into a 0.0995 dm pathlength cell with a thermostated water jacket. The optical rotation at 546 nm was then taken at various temperatures on a Bendix 143A polarimeter. At each temperature the system was allowed to equilibrate before measurements were taken.

Concentrations (Table 3.1) were found as follows. A sample was taken from the cell, weighed, then freeze-dried. The sample was then dried to constant weight in a vacuum desiccator at 100°C and the concentration calculated.

The optical rotation data (Table 3.2) are expressed as the compensated disaccharide rotation (C.D.R).

$$\text{C.D.R.} = \frac{M \cdot \alpha_{546}}{c \cdot l} \cdot K$$

where M is the average disaccharide weight; c is the concentration in g/100 ml; l is the cell pathlength in decimeters; and K is the correction factor discussed above. The measurement of optical rotation versus temperature for amylose was carried out by two methods.

a) Amylose ($\sim$ 0.05g) was added to water (5 ml) and heated in a pressure cooker for 40 minutes. The hot solution was then filtered through a 0.4μ millipore filter into a 0.0995 dm cell and optical rotation measurements taken as usual at 546 nm on a Bendix 143 A polarimeter.

b) [130]Amylose (0.500g) was dispersed in potassium hydroxide (70 ml, 0.5 M) and stirred overnight. The solution was then made up to 100 ml with 0.5 M potassium hydroxide, neutralised with hydrochloric acid, and made

up to 250 ml. The solution was millipored (0.4μ) into a 1 dm cell and optical rotation measurements taken as usual on a Perkin Elmer 141 polarimeter. The results for methods (a) and (b) are shown in Table 3.3 and are the polarimeter measurements uncorrected for refractive index and density.

Optical Rotation of Laminaran in Solvent Mixtures

DMSO-water mixtures of approximately 90% - 10%, 80% - 20% - - - 10% - 90% composition respectively were made up. The exact composition of each was determined from the refractive index of the solvent mixture as this had been found to bear an essentially linear relationship to the solvent composition.

Solutions of laminaran (1% w/v) were made up in each solvent and the optical rotation was measured at 25, 34 and 75°C.

The experiment was repeated at 25°C for DMSO-formamide and water-formamide systems and for 0.5% laminaran in DMSO- water. The results are shown in terms of the compensated specific rotation (C.S.R) on graphs 3.4 - 3.7.

Formation of β-1,3-Glucan Gels

Gels could be formed by two methods. In the first the polysaccharide was dissolved in 0.5 M KOH or DMSO. Subsequent dialysis against running tap water brought about gelation.

In the second method the polysaccharide material was dispersed in a Potter or "Ultraturrax" homogeniser then heated in a water bath (90°C) for 10 minutes.

Molecular Weight of Laminaran

The molecular weight of laminaran in water at 35°C was determined by the Archibald (or approach-to-equilibrium) method. A similar determination was attempted in DMSO but the very small value of the refractive index increment with polymer concentration introduces unacceptable errors.

The ultracentrifuge determinations were carried out in a Beckman Model E analytical ultracentrifuge by Dr. B.G. Newsom of Unilever Research.

The value of the partial specific volume of laminaran in water at 35°C was determined by B.J. Birch of Unilever Research using an Anton Paar density meter (DMA02/C).

C. Results and Discussion

1. Optical Rotation-Temperature Behaviour of Some Polysaccharides

Optical rotation measurements were made against temperature for the following polysaccharides (Table 3.1) :- dextran, inulin, araban, galactan, an extracellular slime polysaccharide from Escherichia coli, bacterial levan and a capsular polysaccharide from Salmonella typhimurium. The results are shown in Table 3.2.

These measurements provide no indication of sharp, sigmoidal optical rotation changes and hence no evidence of gross conformational changes for any of these polysaccharides. In fact the changes in C.D.R. on heating these polymers is of the same order of magnitude as those observed for monosaccharide and disaccharide solutions.[131]

It must be pointed out, however, that the technique employed was not sensitive enough to detect conformational transitions involving little change in the angles ψ and ϕ. It is also possible that the polymer concentration was insufficiently high to allow the existence of an ordered conformation. (This would in fact be the case for a 1% carrageenan system cooled to only 20°C.)

2. Amylose

Much controversy has surrounded the conformation of amylose in neutral solution. If an ordered conformation did exist it might be possible to "melt" it out on heating and follow the transition by optical

rotation. This has been attempted previously [130] and relatively large optical rotation changes were observed.

Table 3.3 shows that optical rotation measurements on heating and cooling amylose in aqueous and salt solution fail to suggest the involvement of a conformational change. Hydrodynamic data[132] and an investigation of the linkage rotation of α-1,4-linked oligosaccharides and amylose [67] indicate that the polymer is likely to be a random coil in neutral aqueous solution. Thus, although the results in Table 3.3 do not preclude the existence of an ordered conformation, they in no way support the claim that significant changes in chain shape are occurring.[130]

3. β-1,3-Glucans

a) General

β-1,3-glucans occur widely in nature and function both structurally and as a carbohydrate reserve. Laminaran, a storage polysaccharide from brown seaweed, has been shown to be a β-1,3-glucan having a chain length of 15-30 residues.[98] Two forms have been identified, namely "soluble" and "insoluble" laminaran. Manners et al [133] have shown that they differ only in their degree of branching, insoluble laminaran being essentially linear whereas soluble laminaran has two or three α-1,6-branch points per molecule. Other β-1,3-glucans with a similar function include paramylon [134] a linear molecule found in the Euglenophyta (single cell flagellates) and chrysolaminaran [135] found in the cytoplasmic inclusions of diatoms.

The structural glucan of the yeast cell wall is thought to be predominantly β-1,3-linked [136] with some branching but the exact structural details are as yet unclear.

Many β-1,3-glucans form aqueous gels. These include yeast glucan,[137] pachyman [138] (from the tree root fungus Porio cocos) and curdlan [139] (an extracellular polysaccharide produced by a mutant of Alcaligenes faecalis var. myocogenes 10C3).

The functions and properties of the β-1,3-glucans would thus suggest the involvement of chain association (gels) and efficient packing (storage). This is likely to involve the existence of ordered chain conformations. At least two other factors indicate the adoption of specific conformations by β-1,3-glucan chains.

(i) Optical Rotation

The optical rotation of most polysaccharides can be obtained by extrapolation of the oligosaccharide series, e.g. α-1,4-glucans ⟶ amylose; α-1,6-glucans ⟶ dextran; β-1,6-glucans ⟶ pustulan (fig. 3.2) [140]. This relationship, known as the Kuhn-Freudenberg relationship, does not hold for the β-1,3-glucan series as the polymer lies off the line (fig. 3.2).

The linkage rotation of the oligosaccharides can be rationalised by allowing for the bias caused by the O(2)-O(2′) hydrogen bond (fig. 3.3 a)[110]. Due to the co-operative nature of hydrogen bonding this influence becomes increasingly pronounced (fig. 3.3 b) as we ascend the oligosaccharide series and accounts for the Λ_D of +33° for the disaccharide and +13°

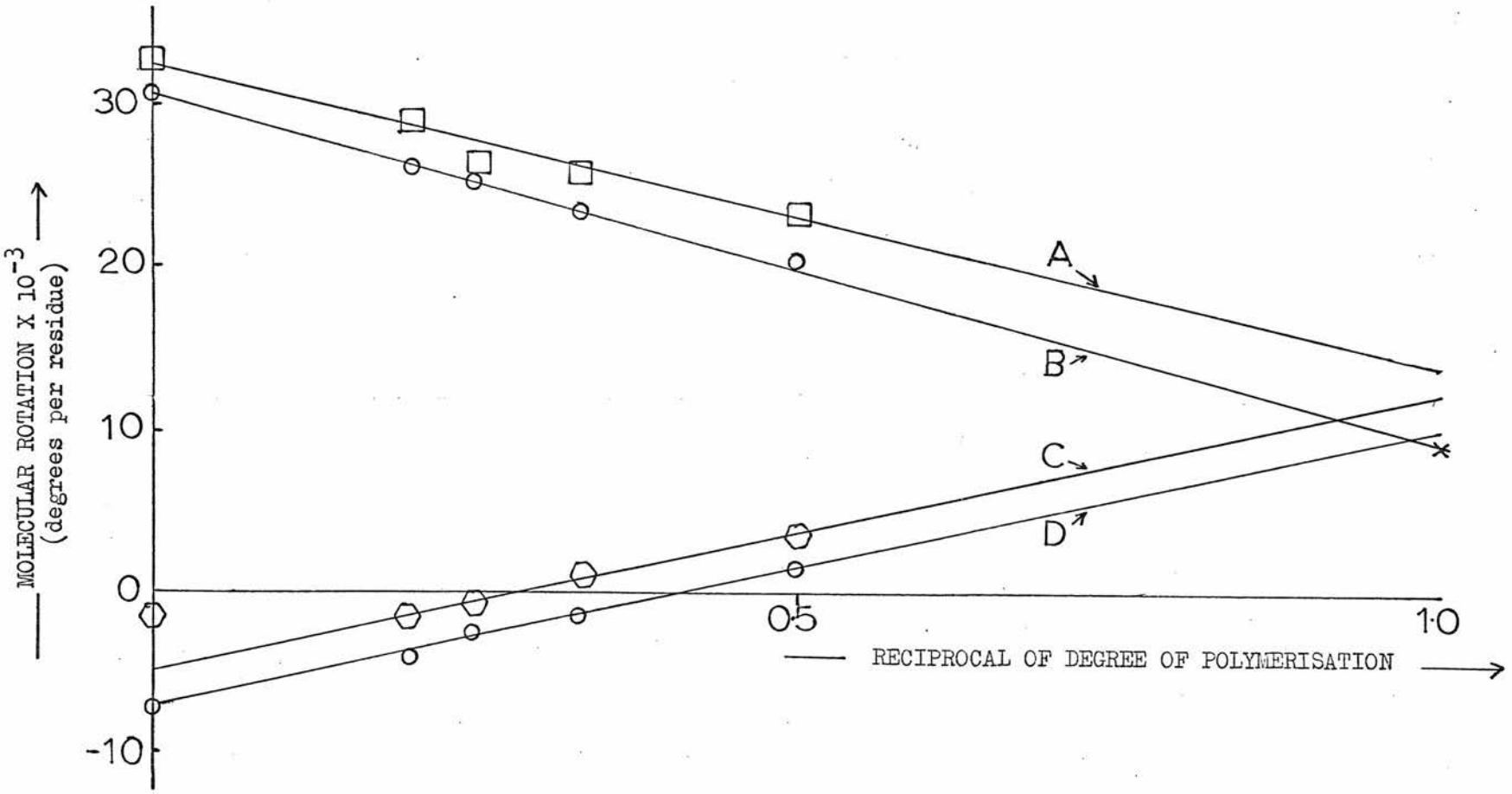


Fig. 3.2. Kuhn-Freudenberg plots for (A) the amylose series, (B) the dextran series, (C) the laminaran series and (D) the pustulan series.

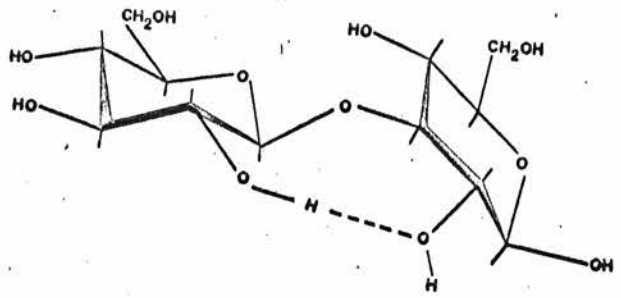


Fig. 3.3 (a). Single O(2) - O(2′) hydrogen bond (shown for laminaribiose).

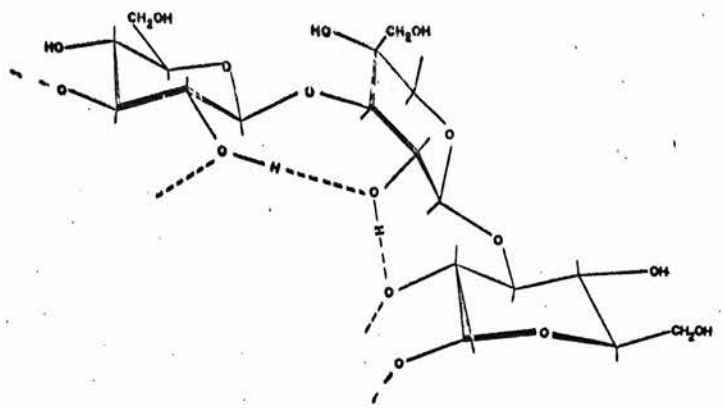


Fig. 3.3 (b). Co-operative O(2) - O(2′) hydrogen bond of β-1,3-glucans (see text).

for the hexasaccharide. Thus for the polymer we would predict a specific rotation of -33° but β-1,3-glucans typically have values around -10°. [141] This must imply that in the polymer either the optical rotation contribution from the individual sugar residues must change or the linkage contribution must change. It has been shown by nmr [142] that the polymer residues are generally in the C1 chair as are those of the oligosaccharides. It would thus appear that the glycosidic bond angles are altered in the polymer suggesting the existence of long range co-operative forces.

(ii) The Solid State Conformation of β-1,3-Xylan

In 1964 Frei and Preston [143] proposed a double helical conformation for a β-1,3-xylan from certain siphoneous green algae. A more recent interpretation [144] of their results, combined with computer model building and infra red dichroism studies, has shown that this polysaccharide exists as a triple helix (fig. 3.4) in the solid state. Each helical chain is thought to be right-handed and has six residues in a pitch of 18.36Å. The structure is stabilised by an interesting triad of hydrogen bonds formed by the O(2) hydroxyl groups which point to the centre of the helix (fig. 3.5).

Preston [40] has pointed out that the early X-ray powder diffraction photographs, which show the crystallinity of β-1,3-glucans are similar to those obtained from β-1,3-xylan. Computer calculations [35] have shown that a xylan-like triple helix is possible for β-1,3-glucans as is the hydrogen bond triad. This is not unexpected as C(6) of all residues in the xylan triple helix lies to the outside. Thus the extra carbon atom on the glucan can project straight out from the helix axis.

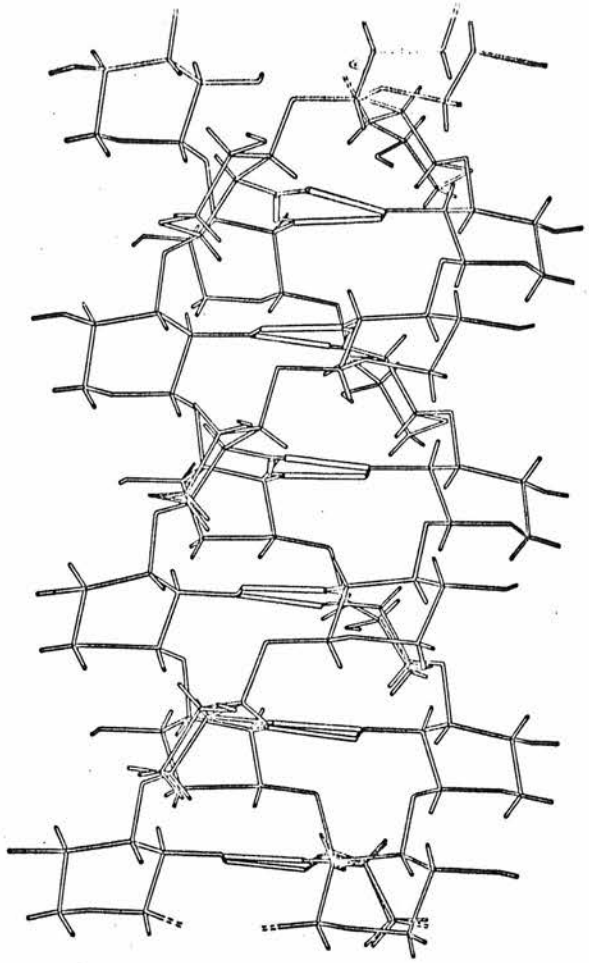


Fig. 3.4. Triple helix proposed for β-1,3-xylan (from ref. 144).

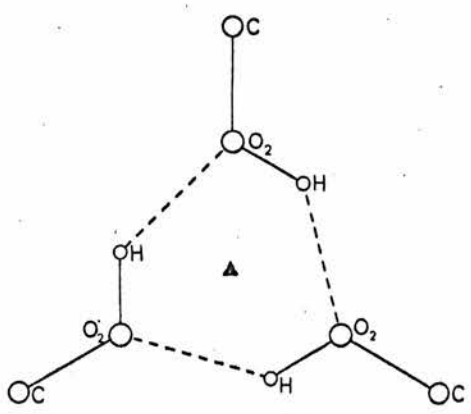


Fig. 3.5. Hydrogen bond triad of the β-1,3-xylan triple helix (from ref. 144).

Recent X-ray diffraction diagrams from fibres drawn from gels of yeast glucan, pachyman and curdlan can be indexed on essentially the same lattice as those of the xylan. [145] These results and observations have led to a proposal [145] for the mechanism of gelation of β-1,3-glucans based on the triple helix functioning as a junction zone as does the double helix in carrageenan. This proposal has also been advanced to account for the network [146] that the glucan is thought to form in the yeast cell wall.

An attempt has now been made to confirm the existence of such a conformation in solution by demonstrating a coil to triple helix transition.

b) (i) Optical Rotation in Water, Dimethylsulphoxide and Formamide

Initially the possibility of a thermally reversible conformational transition was investigated by recording the temperature dependence of the optical rotation of aqueous solutions of insoluble and soluble laminaran. The results were then compared with the temperature dependence in other solvents.

In the case of soluble laminaran (graph 3.2) there is no difference in the overall change of optical rotation with temperature in DMSO or water and in neither case is the transition sharp enough to suggest co-operative conformational changes.

Despite the fact that insoluble laminaran retrogrades from aqueous solution and the higher molecular weight β-1,3-glucans are insoluble in water, all β-1,3-glucans are soluble in DMSO. This indicates that there is no chain association in this solvent and hence that the chains are

likely to be present as random coils. The close similarity of the optical rotation of laminaran in DMSO and water suggests that a similar conformation could also be adopted in water.

For a 0.5% solution of insoluble laminaran the results are essentially the same (graph 3.1). In more concentrated solutions, however, the picture changes. The behaviour in DMSO remains unchanged but in water the optical rotation reaches a minimum (at 30°C in a 1% solution and 45°C in a 2% solution) then starts to rise. Below these temperatures the polysaccharide often precipitates from solution in a manner which is very similar to the retrogradation of amylose. Optical rotation data obtained on solutions at low temperature ($<10^{\circ}C$) appeared to follow the upward trend but some difficulty was experienced in judging whether this change was based on changes in the chain geometry or was merely brought about by artifacts introduced by particle formation or misting of the cell endplates. All points on the curves shown on graph 3.1 were, however, stable for several days.

A comparison with the optical rotation-temperature behaviour of laminaribiose in water (graph 3.3) shows that the change for the polymer is of the same order of magnitude as for the disaccharide. Nmr spectra of laminaribiose were recorded at various temperatures to demonstrate that the ratio of the α to β anomer did not change significantly on heating and hence could not be influencing the optical rotation changes for the disaccharide.

(ii) Optical Rotation in Mixed Solvent Systems

If laminaran exists in an ordered conformation in water and a disordered conformation in DMSO, then a graph of optical rotation against

the percentage composition of a series of DMSO-water solvent mixtures will show a sharp sigmoidal change. The same should be true in formamide-water systems but in formamide-DMSO systems, since no ordered conformation is expected, the change from one solvent to the other will show a more gradual transition.

In DMSO-water systems the results (graphs 3.4 and 3.5) are difficult to interpret but the differing shape of the 0-20% DMSO regions of the curves at 25, 34 and 75°C can be related to the effect which causes the results in water to show a minimum.

At low temperature laminaran precipitates from solution in solvents containing about 60-70% DMSO. This is the region where the two solvents are known to interact strongly with each other and it is probably this "competition" for solvent that results in the insolubility of the material. It has been shown [145] by X-ray powder diffraction that the material precipitated in this system is amorphous whereas the material obtained on retrogradation from water is crystalline.

The graph for 0.5% insoluble laminaran in DMSO-water mixtures at 25°C has the same shape as a 1% solution. In fact the shape of the curve in the 0-20% DMSO region suggests that the effect which causes the 1 and 2% solutions to show a minimum in water is still present at 0.5% but is not so pronounced.

The overall shape of the curves and their magnitude and sharpness do not favour a co-operative conformational transition.

In DMSO-formamide the change between solvents is gradual. Formamide-water systems are essentially linear with respect to optical rotation.

Thus we have no evidence for a conformational change on heating 0.5% aqueous solutions of laminaran from 25°C. However, cooling below 30°C for a 1% solution or 45°C for a 2% solution may involve the formation of some material in an ordered form which is stable prior to precipitation. This is supported by the crystalline nature of the precipitate.

c) The Molecular Weight of Laminaran

If laminaran exists in water as a triple helix (or any other aggregate) then the molecular weight in aqueous solution should indicate a higher degree of polymerisation (D.P.) than in DMSO or than that obtained by end group analysis and similar techniques.

The molecular weight of laminaran has been estimated by many techniques (Table 3.4) all of which indicate a molecule of about 20 glucose residues. However, Broach and Greenwood have suggested that laminaran is polydisperse.[147]

The particle weight determined by ultracentrifugation, as described in the experimental section of this chapter, gave a value for the molecular weight in water as 4315 ± 15 or a D.P. of about 27 units.

The refractive index increment between DMSO and a 1% solution of laminaran in DMSO was too low (0.0002 ± 0.0001 compared with 0.0015 ± 0.0001 in water) to allow a meaningful molecular weight to be obtained by the

method employed. However it is obvious that the value in water does not represent a triple helix with a D.P. about 60.

d) Formation of β-1,3-Glucan Gels

As described in the experimental section gels were formed from curdlan, pachyman and sclerotan in two ways. Gels obtained by dispersion and heating were milky white, broke up on heating and after prolonged heating hard white lumps were formed and liquid separated from the gel. Gels formed from dialysis were less opaque but once again hard white lumps were formed on heating. In both cases gels were hard and brittle and syneresis was very rapid.

On account of the structural similarity of β-1,3-glucan and β-1,3-xylan attempts were made to gel a β-1,3-xylan from Caulerpa filiformis. No gels were formed however by either method, dialysis from alkali or DMSO merely resulting in precipitation.

Sclerotan, a β-1,3-glucan with β-1,6-stubs on every third residue, also forms gels by the above methods but the gels obtained are less opaque. This might be expected as a substituent on C(6) will not prevent formation of the triple helix but could hinder aggregation of these helices.

The opaque nature of β-1,3-glucan gels and the tendency of laminaran to aggregate suggests that, as in kappa-carrageenan and agarose systems, helix aggregation may be an important factor in gel formation and properties. In fact the behaviour of the gels on prolonged heating suggests that a more and more stable aggregate is being formed, perhaps by annealing.

In fact, dispersion and heating methods of gelation need not involve a break up of the triple helix but only a rearrangement of aggregates.

e) β-1,3-Glucan Conformation

$\Delta \phi$ and $\Delta \psi$ (fig. 3.1) have been obtained for the β-1,3-glucan triple helix from X-ray fibre diffraction.[145] Assuming the residue co-ordinates of Arnott and Scott[148] the values obtained for the right-handed triple helix, $\Delta \phi = 31.2 \pm 1.5^{\circ}$ and $\Delta \psi = 9.0 \pm 0.5^{\circ}$, correspond to a linkage rotation of $24 \pm 3^{\circ}$ and a specific rotation of $-26 \pm 2^{\circ}$. Thus the value of -8° to -12° obtained for laminaran (graph 3.1) cannot be explained by the formation of this ordered conformation. The X-ray and computer model building evidence for the β-1,3-glucan triple helix, or the β-1,3-xylan triple helix, does not preclude the left-handed form, $\Delta \phi = 12.8 \pm 0.5^{\circ}$ and $\Delta \psi = -33.4 \pm 1.5^{\circ}$, but the corresponding specific rotation $82.5 \pm 1.5^{\circ}$ is even further from the observed value for laminaran. However, it does raise the possibility that the specific rotation of laminaran could be explained by the existence of both this ordered form and the random coil at the same time. Thus a value of -10° could represent 80% of the molecules being present as the random coil ($[\alpha]_D = -33^{\circ}$) and 20% as the left-handed triple helix ($[\alpha]_D = +84^{\circ}$). Such a situation would be possible if only a fraction of the molecules had sufficiently long chain regions capable of forming the triple helix at the temperature studied: a situation for which there is some evidence.[147]

This 20% helix/80% random coil mixture is not favoured by the ultracentrifuge measurements as only a single peak was observed. This suggests that the anomalous specific rotation of the polymer must be the

result of some other effect. Loss of the co-operative O(2) - O(2′) hydrogen bond could account for the anomaly but no reason can be advanced to explain why such a favourable hydrogen bonding structure should be broken down at longer chain length. Computer model building studies have shown that this co-operative hydrogen bonding system is not sterically hindered in long chains, thus the specific rotation anomaly remains unexplained.

The changes in optical rotation of laminaran solutions at low temperature suggest that, if any ordered conformation is being formed prior to retrogradation, it is likely to be the left-handed triple helix (values to + 84° not - 26°). If the formation of such a triple helix occurs in insoluble, but not soluble, laminaran it could account for the differing solubility of the polymers as the soluble form would not be able to adopt the rigid rod necessary for retrogradation.

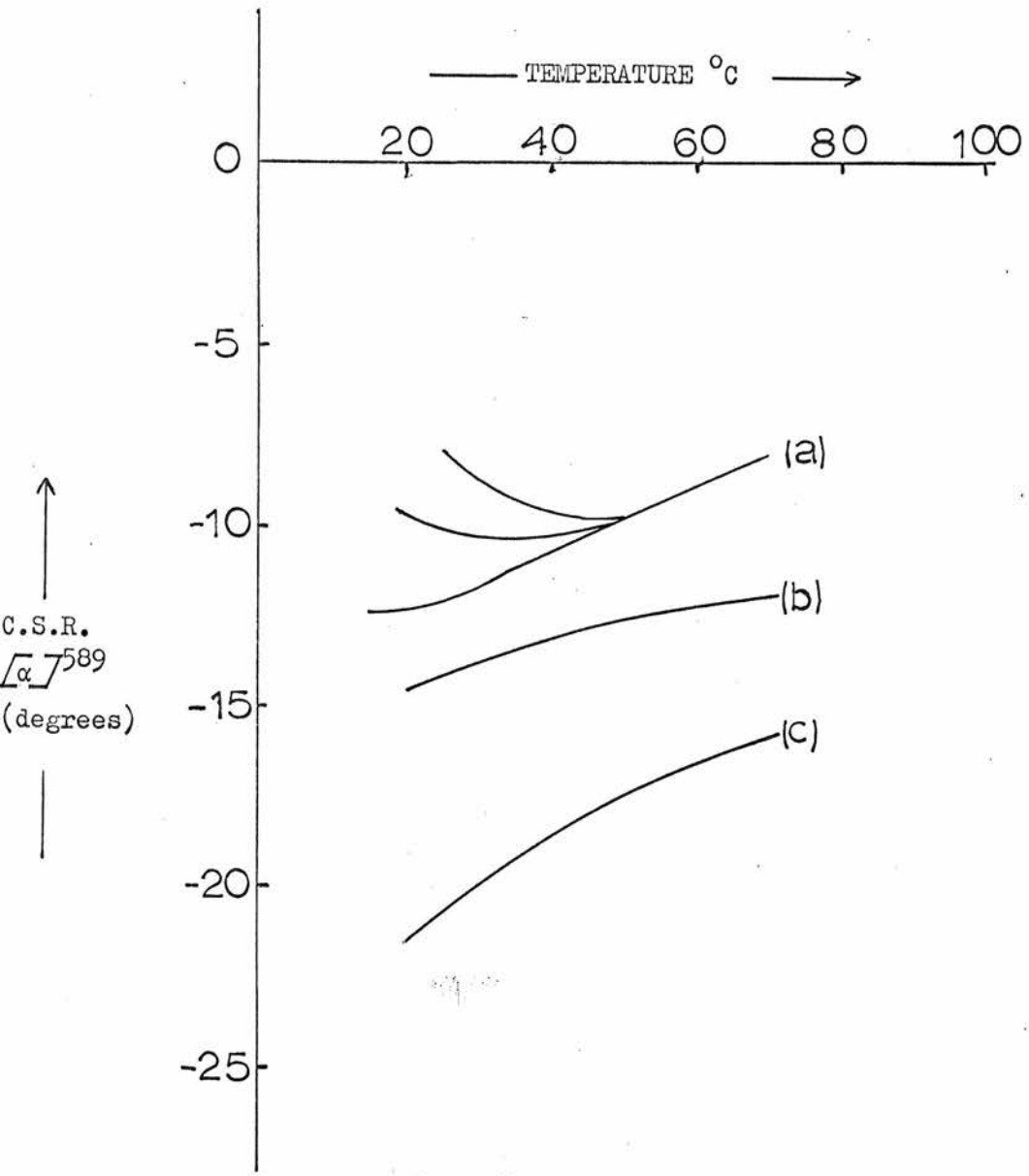
Very recently, since the above was completed, measurements of optical rotation, viscosity and flow birefringence have been employed to demonstrate that curdlan exists as an ordered structure in sodium hydroxide below 0.19M and as a random coil in sodium hydroxide above 0.24M. [149] These studies have shown that a sharp transition occurs between 0.19 and 0.24M alkali.

Ogawa et al [150] have also shown that the dye Congo Red binds to this β-1,3 glucan when in the ordered conformation and displays similar behaviour to the carrageenan/methylene blue system including stabilisation of the ordered conformation by the dye molecules (the transition indicated by the dye occurs between 0.22 and 0.25M sodium hydroxide).

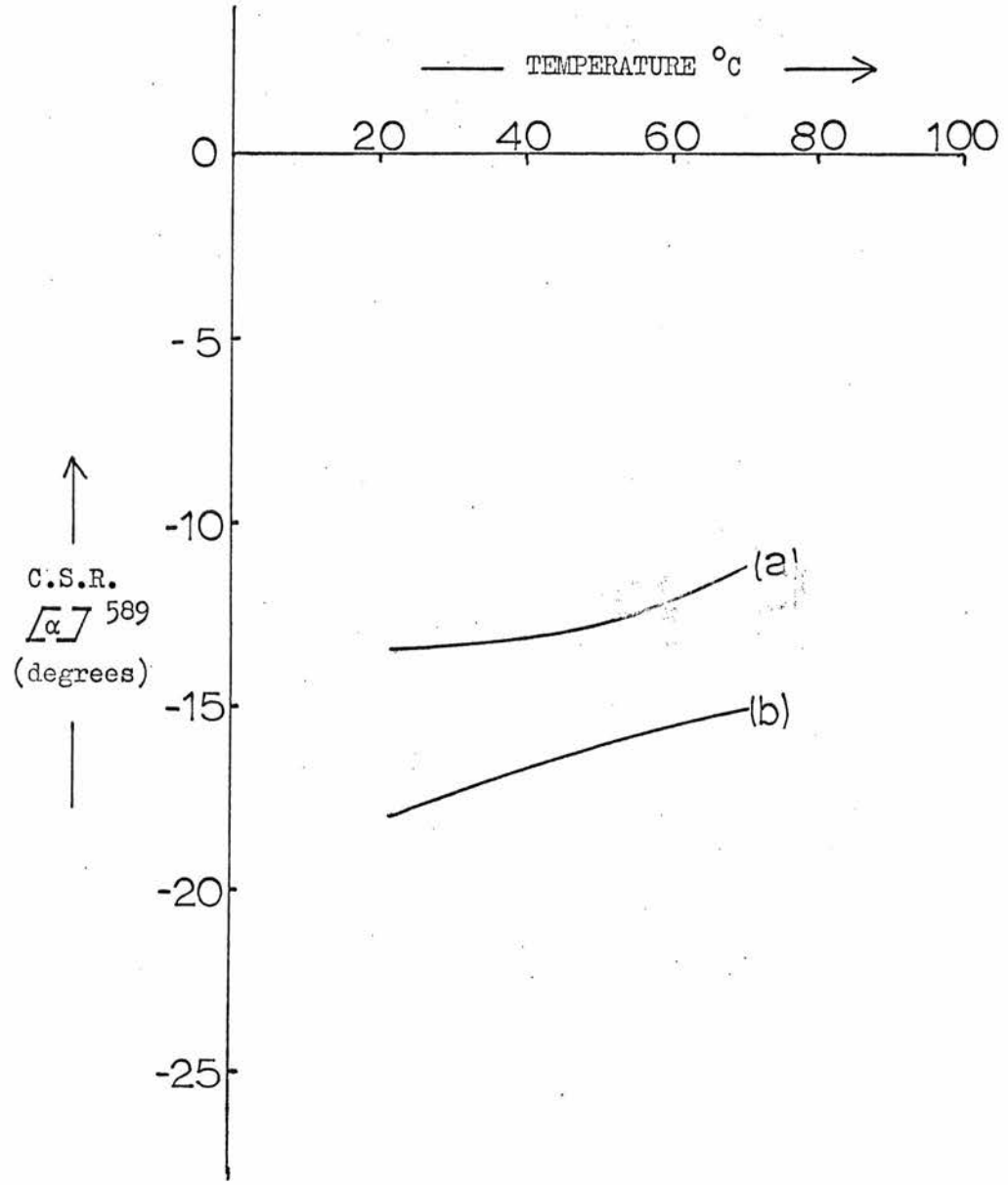
Although the authors describe an amylose-like single helix as the likely ordered conformation the value of the specific rotation of the glucan in low alkaline solution is close to the value of + 82.5° predicted for the left-handed triple helix. As the specific rotation drops sharply to about 0° at 0.24M alkali I suggest that they are in fact observing the triple helix to coil transition. These studies also show that degraded material of D.P. 17 does not appear to form the ordered conformation. This may be relevant to the conformation of laminaran (D.P. 20).

The evidence for the triple helix in the gel and glucan network is thus fairly substantial but the optical rotation and ultracentrifugal behaviour of laminaran cannot be completely explained by this conformation. The optical rotation data of Ogawa et al favours the formation of the left-handed triple helix in curdlan and such a conformation could be formed, to a limited extent, by laminaran at low temperature in aqueous solution.

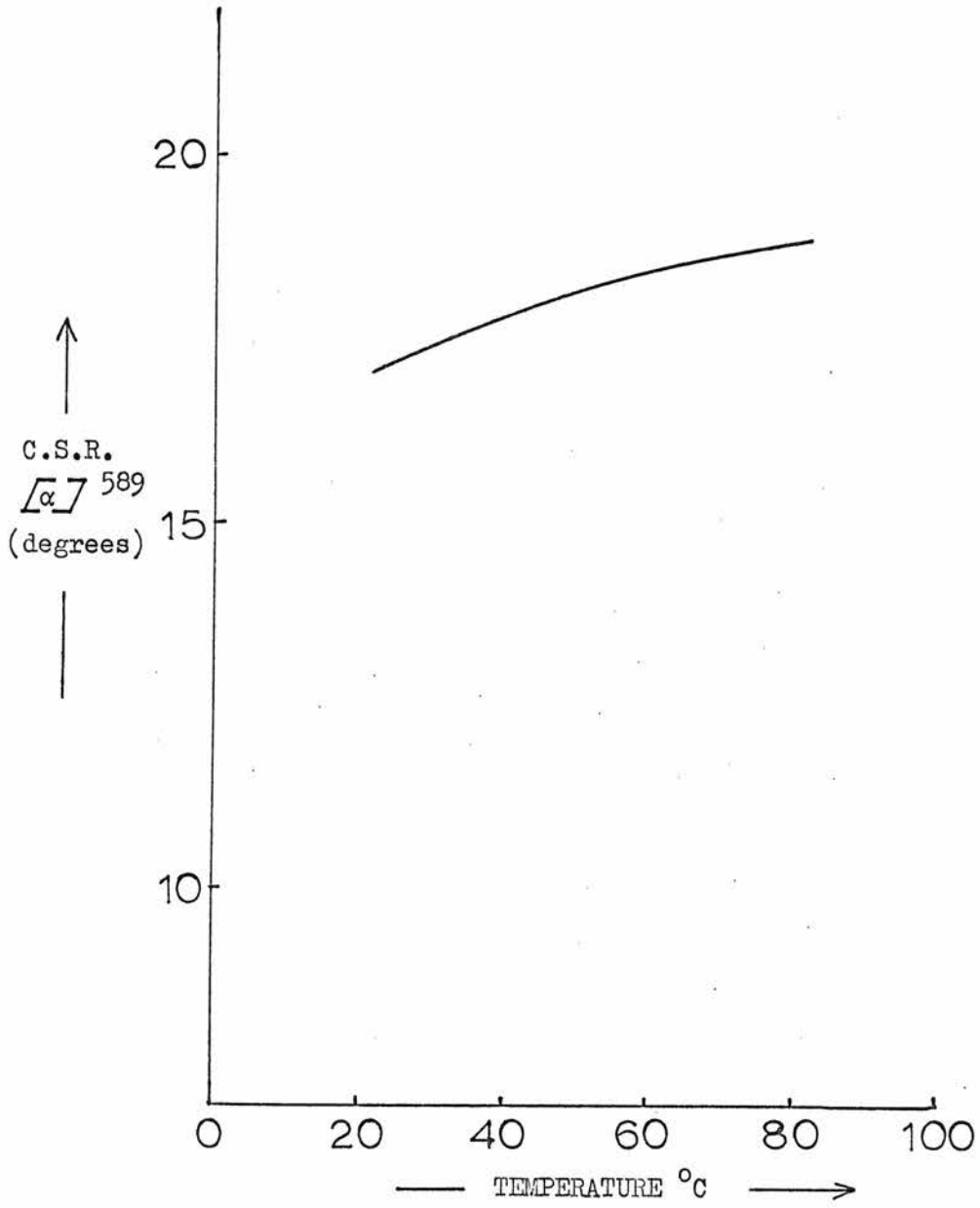
D. GRAPHS AND TABLES



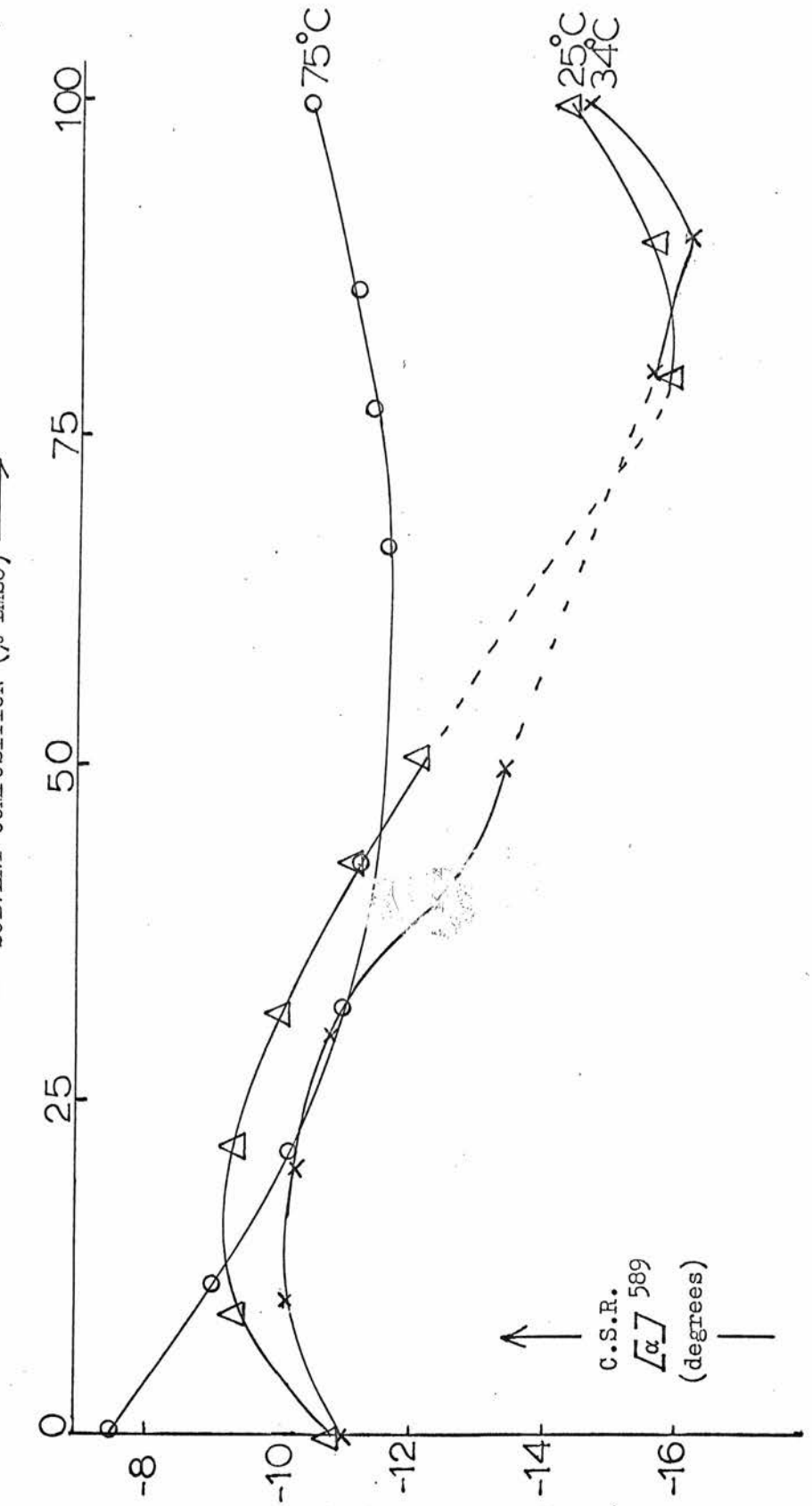
Graph 3.1. Variation in compensated specific rotation (C.S.R.) on heating and cooling solutions of insoluble laminaran in (a) water (0.5, 1.0 and 2.0%), (b) DMSO (1%) and (c) Formamide (1%). In the formamide case no correction is made for refractive index or density changes.



Graph 3.2. Variation in compensated specific rotation (C.S.R.) on heating and cooling solutions of soluble laminaran in (a) water (1%) and (b) DMSO (1%).

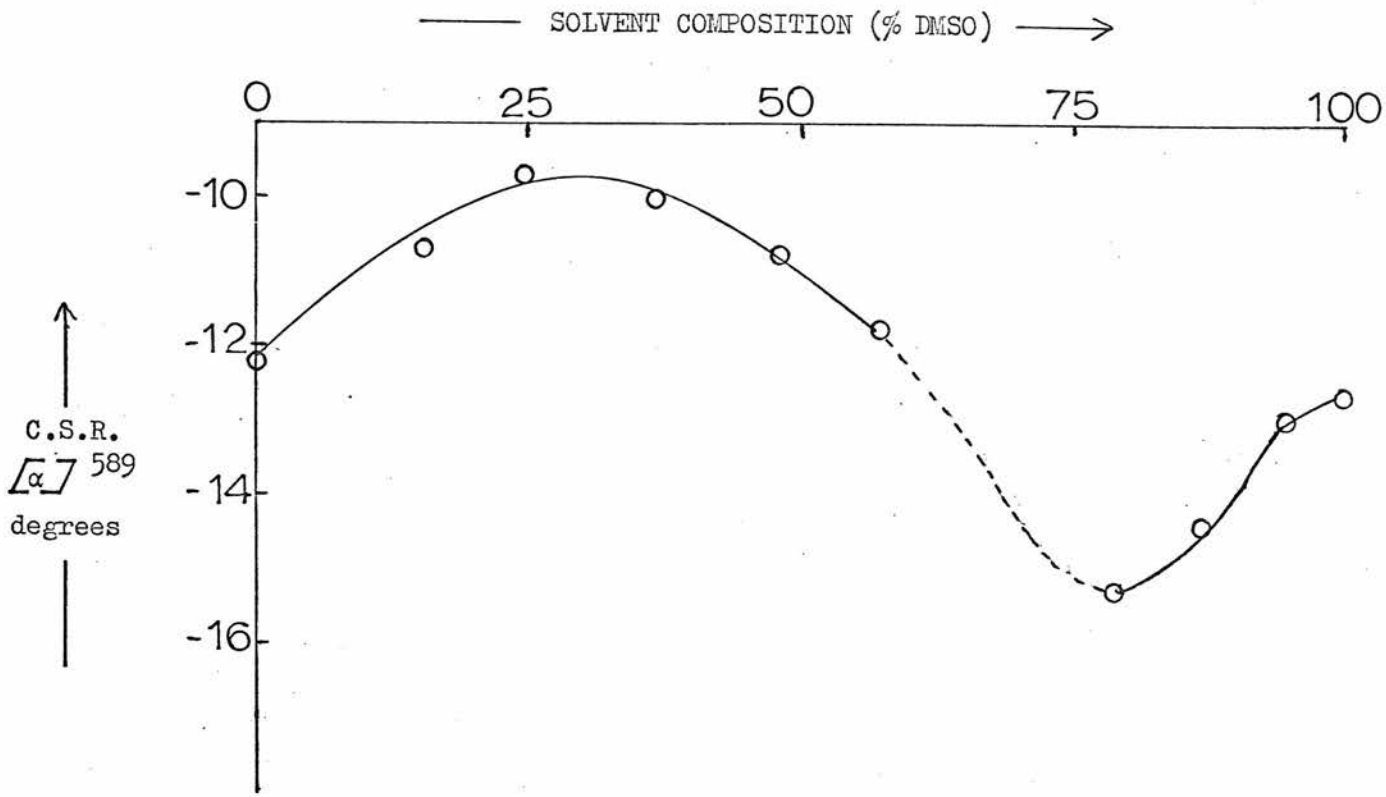


Graph 3.3. Variation in compensated specific rotation (C.S.R.) on heating and cooling an aqueous solution of laminaribiose.

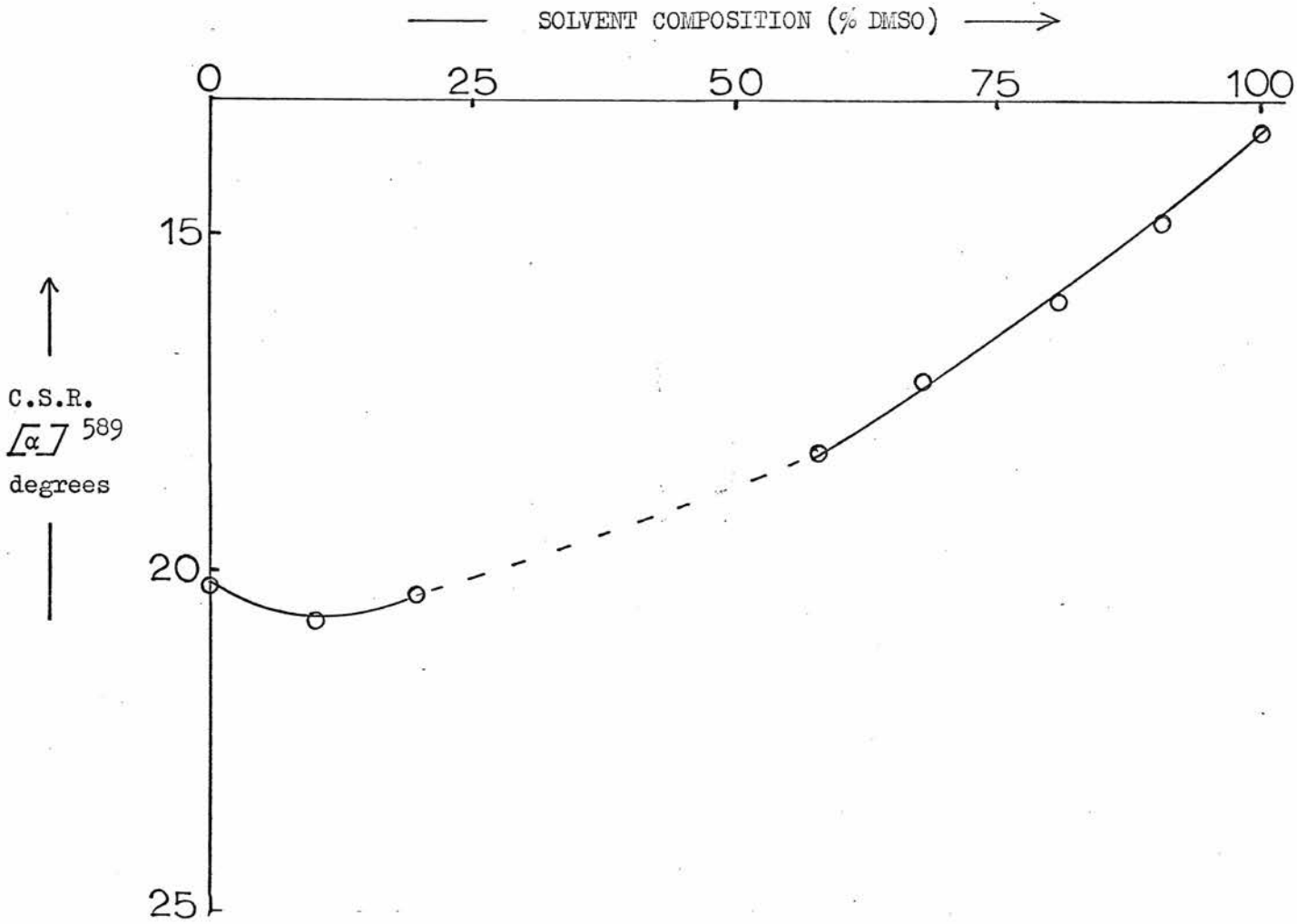


Graph 3.4. Variation in compensated specific rotation (C.S.R.) with solvent composition for insoluble laminaran (1%) in DMSO - water mixtures at 25°C, 34°C and 75°C.

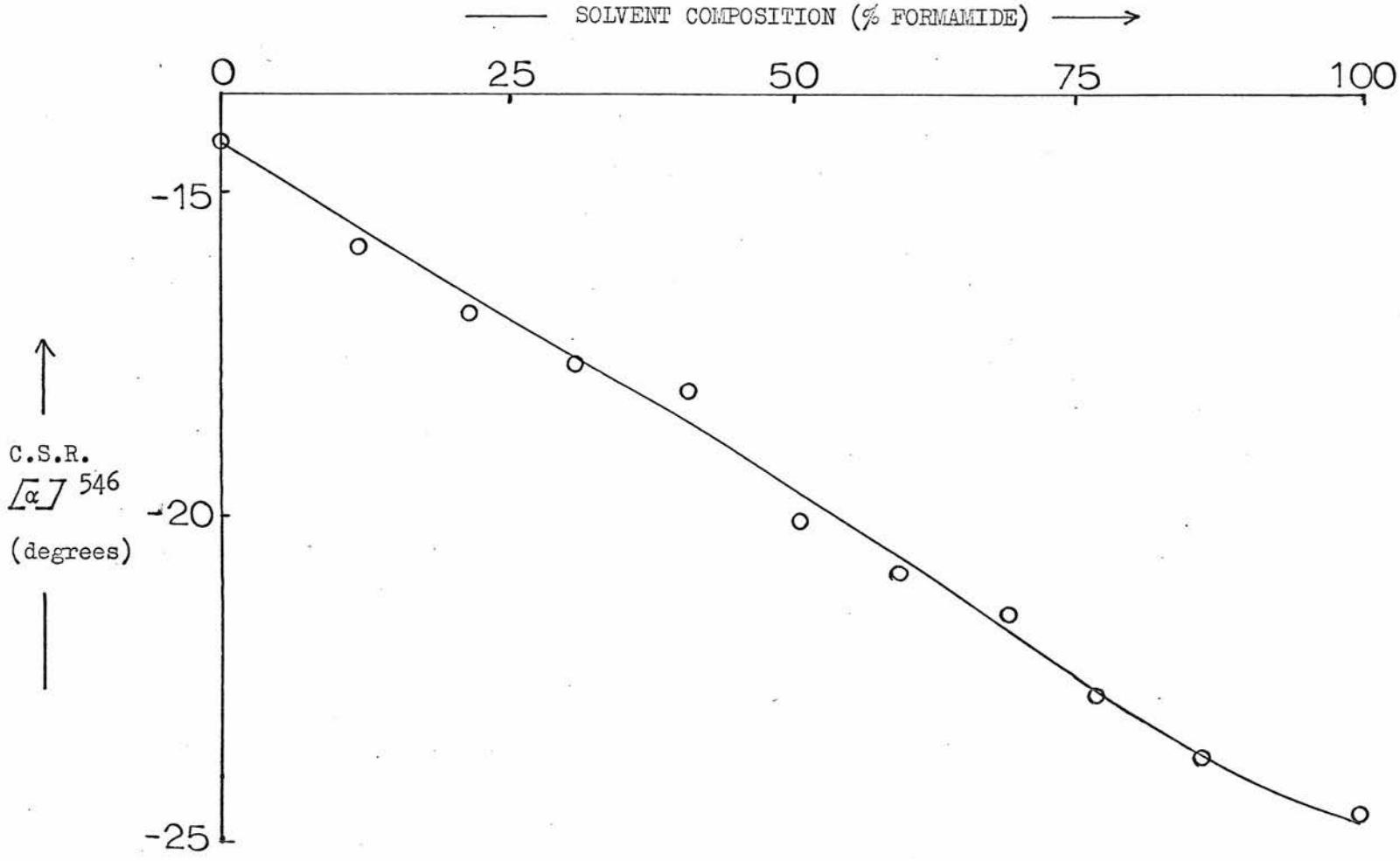
Corrections are made to 100% aqueous solution.



Graph 3.5. Variation in compensated specific rotation (C.S.R.) with solvent composition for insoluble laminaran (0.5%) in DMSO - water mixtures at 25°C. Corrections are made to 100% aqueous solution.



Graph 3.6. Variation in compensated specific rotation with solvent composition for insoluble laminaran (1%) in DMSO - formamide mixtures at 25°C. Corrections are made to aqueous solution at 25°C.



Graph 3.7. Variation in compensated specific rotation (at 546 nm) with solvent composition for insoluble laminaran (0.5%) in water - formamide mixtures at 25°C. Corrections are made to 100% aqueous solution.

Table 3.1

POLYSACCHARIDE	TIME OF HEATING (mins)	FILTER (μ)	CONCENTRATION (g/100 ml)
Dextran	20	0.2	0.7
Inulin	25	none	1.0
Araban	5	none	0.9
S.typhimurium capsular polysaccharide	25	1.2	0.3
Galactan	25	0.4	1.0
Bacterial levan	25	1.2	1.0
E.coli slime polysaccharide	25	1.2	0.8

Table 3.2. The optical rotation/temperature behaviour of several polysaccharides.

DEXTRAN		INULIN		ARABAN		S.TYPHIMURIUM CAPSULAR	
T	C.D.R.	T	C.D.R.	T	C.D.R.	T	C.D.R.
69	344	69	75	71	75	70	13 ± 7
60	341	60	75	60.5	74	60	13 ± 7
49.5	344	50	74	49.5	74	51	13 ± 7
40	342	40	76	40	73	40	7 ± 7
30	345	31	75	30	73	30	10 ± 7
20	346	21	77	20	73	21	7 ± 7

GALACTAN		LEVAN		E.COLI SLIME	
T	C.D.R	T	C.D.R.	T	C.D.R.
70.5	90	70	90	70	44
58.5	90	60	90	60.5	44
50	89	50	91	50	44
40	89	39	90	40	43
30	92	28	91	30	43
20	93	21	91	21	43

T is the temperature in ^{o}C and C.D.R. is the compensation disaccharide rotation in degrees at 546 nm.

The lack of clarity and the low values of optical rotation for the capsular polysaccharide from S. typhimurium resulted in the error ($\pm 7^{o}$) obscuring any changes which may have occurred.

Table 3.3 The optical rotation/temperature behaviour of amylose in (a) aqueous and (b) salt solution.

(a)

TEMPERATURE (°C)	OPTICAL ROTATION (degrees at 546 nm)
86	0.044
73	0.044
59	0.044
44	0.046
29.5	0.046
20	0.047

(b)

TEMPERATURE (°C)	OPTICAL ROTATION (degrees at 546 nm)
76	0.107
68	0.108
56	0.109
35	0.110
24	0.111

Table 3.4 Molecular Weight and Chain Length of Laminaran

Estimated Molecular Weight	Chain Length, anhydro-D-glucopyranose units	Soluble (S) or Insoluble (I) Laminaran	Method
5000-6000		I	Iodine absorption
3514	21-22		Cryoscopic
	16	I	Periodate oxidation
2600-3500	13-17	I[a]	Microisopiestic method
3500-5000			Osmotic pressure
about 2600			Viscosity
	20		Yield of 2,3,4,6-tetra-O-methyl-D-glucose on hydrolysis
2800-3800	14-18	S[a]	Microisopiestic method
about 5800[b]		I S[b]	Viscosity
	20	S[a]	Yield of 2,3,4,6-tetra-O-methyl-D-glucose on hydrolysis
3500	21	I	Sedimentation, diffusion
5300	33	S	and viscosity measurements
4130	about 25		Hypochlorous acid/sodium phenate with Kiliani cyanhydrin reaction
1900	9	[a]	Isothermal distillation method
	17		Sodium borohydride reduction, periodate oxidation followed by estimation of formaldehyde
	about 19	S	Oxidation followed by estimation
	about 24	I	of formaldehyde after dialysis

[a] Methylated laminaran [b] Acetyl laminaran

Materials

Kappa-carrageenan. Two samples were used. The first was prepared from Chondrus crispus and the second from Eucheuma cottonii. Both were prepared in the laboratory of Marine Colloids Inc., Rockland, Maine 04841, U.S.A. Infrared analysis indicates that the material from Chondrus crispus (code REX 5401) has a high percentage (30-35) of the 3,6-anhydrogalactose residues 2-sulphated, whereas only 5% of these residues, at most, are 2-sulphated in the material from Eucheuma cottonii. Iota-carrageenan was prepared from Agardiella tenera (code RENJ 5223) by Marine Colloids Inc.

Lambda-carrageenan (code RENJ 5263) was prepared from Gigartina canaliculata by Marine Colloids Inc.

All carrageenan samples were used in the potassium salt form.

Agarose (code REX 5468) was provided by Marine Colloids Inc.

Locust Bean Gum was a purified grade purchased from Köbenhavns Pectinfabrik, Copenhagen, Denmark.

Tara Gum was purified material from Marine Colloids (code REX 5922)

Guar Gum was a purified grade purchased from Köbenhavns Pectinfabrik.

Fenugreek Gum was prepared from the seeds of Trigonella foenum-graecum supplied by Marine Colloids Inc. The galactomannan was extracted by R. Moorhouse by the method of Andrews, Hough and Jones.[151]

Tamarind Seed Mucilage was purchased from T.M. Duché & Sons, (U.K) Ltd.

Gleditsia Galactomannan was extracted from Gleditsia thriacantos by Dr. A.S. Cerezo at the University of Buenos Aires.

Carboxymethyl Cellulose (code P8) was obtained from British Cellanese Ltd.

Sapote Gum was purchased from Stein, Hall and Co., Inc., New York.

Corm-sac polysaccharide from Watsonii pyrimidata was provided by Prof. A.M. Stephen of the University of Cape Town.

Insoluble Laminaran (code IL32), prepared from Laminaria hyperborea in 1955, was provided by Dr. E. Dewar of Inveresk Research International, Musselburgh, Midlothian. This material was dissolved in water, filtered and freeze dried to give the material used in the work described.

Soluble Laminaran (code SL5) was provided by Dr. J.J. Marshall of the University of Miami, Florida.

Pachyman was a commercial sample of Korean origin.

Curdlan was provided by Dr. T. Harada of Osaka University, Japan.

Sclerotan was provided by Dr. J.S.D. Bacon of The Macauley Institute for Soil Research.

β-1,3-Xylan was provided by Dr. Elizabeth Percival of Royal Holloway College, University of London, and was isolated from Caulerpa filiformis.

Laminaribiose was provided by Dr. J.R. Turvey of University College of North Wales, Bangor.

Amylose (molecular weight 150,000) was purchased from Koch-Light Laboratories Ltd.

Dextran (code NCIB2786) was obtained from Dr. D.H. Hutson.

Inulin (from dahlia tubers) and galactan (from Lupinus albus) were provided by Prof. Sir Edmund Hirst.

Beet Araban was provided by Prof. G.O. Aspinall.

E.Coli slime polysaccharide and S. typhimurium capsular polysaccharide were provided by Dr. I.W. Sutherland of the University of Edinburgh.

Bacterial levan was that described in reference 152.

Preparation of Polysaccharide Solutions for O.R., U.V., and C.D.

Unless otherwise stated polysaccharides were dispersed in water and then heated in sealed tubes in a pressure cooker at 122°C for 20-30 minutes. The solutions were then filtered hot through a 1.2μm millipore filter. Non-aqueous solutions were filtered through 5μm teflon millipore filters, as D.M.S.O., formamide and alkali were found to attack filters made from cellulose esters.

In the UV and CD experiments using methylene blue the polysaccharide samples were dialysed to prevent interference by salts and here again Teflon millipore filters (5μm) were used as the usual cellulose ester filters were found to retain the methylene blue.

Optical Rotation Measurements

These were carried out on a Perkin-Elmer 141 polarimeter using 1 cm cells for all solutions containing carrageenan and 10 cm cells for the laminaran solutions. Readings were taken at several wavelengths as: readings were amplified at 365 and 436 nm; high light intensities at 436 and 546 nm were useful when transmission was poor; measurements at 589 nm can be compared with literature values. Temperature control was via a circulating water bath with a contact thermometer. The temperature of the water in the cell jacket would be controlled to $\pm$ 0.25°C over the temperature range 10-90°C for a period of at least 24 hours. Sufficient time was allowed for the sample to achieve equilibrium, the final measurement being taken when no change occurred over 20 minutes.

Variations of optical rotation inherent in the cell were allowed for by measurement of cell blanks (the O.R. reading when the cell is filled with distilled water).

Recording of Ultraviolet and Circular Dichroism Spectra

Temperature control was achieved with the same system as in the O.R. measurements. U.V. spectra were recorded with a Unicam SP 800 spectrophotometer using a 1 cm pathlength cell in a thermostated cell holder.

C.D. Spectra were recorded on a Cary 61 spectropolarimeter using a 0.1 cm pathlength cell. Cell blanks were allowed for.

Determination of Gel Melting and Setting Points

After finding the approximate setting point the gel was melted and the temperature of the sol lowered by steps of 2°C with equilibration for 30 minutes at each stage. Glass beads (1.3 ± 0.1 mm diameter) were then introduced just below the surface and the setting temperature was taken to be the point at which these beads failed to sink.

Melting points were determined, after allowing several hours for ageing of the gel, by heating in steps of 2°C as before until the glass beads, which remained from the determination of the gel setting point, just commenced to fall.

Phenol Sulphuric Acid Estimation for Carbohydrate in Smith-Degraded Kappa-Carrageenan

Aqueous phenol (1 ml:5% W/V) was added to a 2 ml portion of the solution (maximum of 0.1 mg carbohydrate/ml of solution). 5 ml concentrated sulphuric acid was added quickly. When the solution had cooled the optical density was measured using an EEL colorimeter (filter 623). The unfractionated polysaccharide was used as a standard.

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APPENDIX

Dea, I.C.M., McKinnon, A.A., and Rees, D.A. (1972). J. Mol. Biol., 68, 153.

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Tertiary and Quaternary Structure in Aqueous Polysaccharide Systems which Model Cell Wall Cohesion: Reversible Changes in Conformation and Association of Agarose, Carrageenan and Galactomannans

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Tertiary and Quaternary Structure in Aqueous Polysaccharide Systems which Model Cell Wall Cohesion: Reversible Changes in Conformation and Association of Agarose, Carrageenan and Galactomannans

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Certain polysaccharides which form gels by a mechanism that is known or suspected to involve the cross-linking of molecular chains in multiple helix formation have been subjected to selective chain-cleavage by Smith degradation at helix-terminating residues. The products retain the ability to convert to helix but do not now form a network and gel properties are thus abolished. Addition of certain galactomannans to such fragmented products from agarose and κ-carrageenan can evidently re-establish the network because characteristic bulk properties re-appear. Similar interactions can be demonstrated with the native (non-fragmented) polysaccharides.

These systems have been studied by optical rotation and its temperature dependence, by interactions with dye using ultraviolet and circular dichroism spectroscopy, and by methods based on chemical fractionation and analysis. It is shown that ordered binding can occur between the agarose or carrageenan helix and parts of the galactomannan backbone that contain contiguous unsubstituted mannose residues. This association of unlike polysaccharide entities is regarded as "polysaccharide quaternary structure" and its influence can illustrate "ligand induction of polysaccharide tertiary structure."

Stereochemical analogies between the galactomannan backbone and polysaccharides such as cellulose and peptidoglycan, suggest that the binding might mimic biological cohesion between skeletal and gel phases of natural cell walls.

1. Introduction

In biological systems, polysaccharides often exist in organized or semi-organized heterogeneous assemblies as, for example, in the plant and bacterial cell walls and the starch granule. We have argued (Rees, 1969*a*; Rees & Scott, 1971; Grant, McNab, Rees & Skerrett, 1969; Lawson & Rees, 1970) that an understanding of non-covalent associations between like chains (tertiary structures) and between distinct, unlike chains (quaternary structures) would help to show how polysaccharides function in such systems. Some progress has been made by the investigation of chain geometry and associations in the solid (fibrous) state using X-ray diffraction and infrared dichroism (Anderson, Campbell, Harding, Rees & Samuel, 1969; Atkins & Parker, 1969). We now describe a complementary method of investigation based on the properties of aqueous thermally reversible polysaccharide gels. This type of approach

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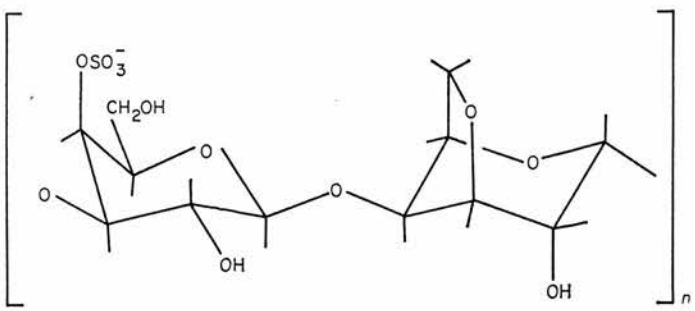
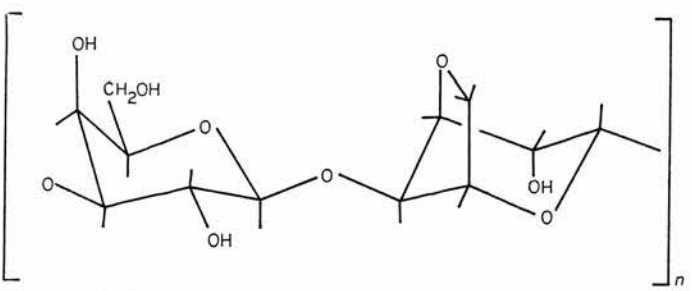
has been used before, especially with gelatin gels to investigate the polypeptide associations that are now known (Flory & Weaver, 1960; Traub & Piez, 1971) to occur in the collagen triple helix: Boedtker & Doty (1954) used the sharp setting and liquefaction behaviour as evidence for the ordered character of these associations; by means of van't Hoff's equation; Eldridge & Ferry (1954) attempted to estimate "heats of association" from the variations of liquefaction temperature with concentration and with molecular weight; Flory & Weaver (1960) measured the kinetics of chain association in an attempt to derive the mechanism of the process; and Tobolsky (1955) used the decay of optical birefringence in various stressed gells to deduce the order of lifetime and hence likely character of the associations present.

The covalent structure of two gelling polysaccharides, κ-carrageenan and agarose, are shown in formulae I and II respectively (for a review see Rees, 1969*a* but see also



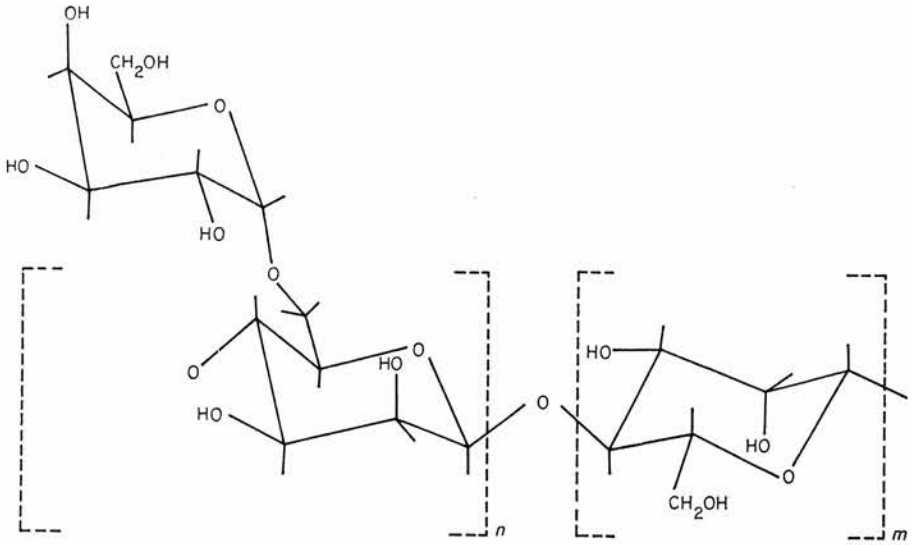
I κ-Carrageenan (idealized)



II Agarose (idealized)

Duckworth, Hong & Yaphe, 1971 and references cited there). There is good evidence that κ-carrageenan is double-helical in the solid state (Anderson *et al.*, 1969). The conformation of agarose has not yet been characterized but striking analogies exist with carrageenans to suggest similarity of conformation. This problem is discussed further in section 5.

The galactomannans of several leguminous seeds are also used in this investigation, especially those from locust bean or carob bean (*Ceratonia siliqua*), tara (*Caesalpinia spinosa*), guar (*Cyamopsis tetragonolobus*), and fenugreek (*Trigonella foenum-graecum*). The covalent structures are based on a β-1,4 linked mannan backbone to which are attached α-D-galactopyranosyl residues as 1,6 linked single-unit side chains (Smith & Montgomery, 1959). Evidence from analysis of the products of enzymic hydrolysis (Courtois & Le Dizet, 1966, 1970) would indicate that galactose substituents tend to occur in blocks with few in isolated positions. To an approximation, therefore, the structures



III Typical galactomannan (idealized)

may be shown as formula III. The proportion of galactose and mannose residues vary from one source to another as indicated in section 2(a). The polysaccharides will here be named galactomannan A, galactomannan B, galactomannan C and galactomannan D, corresponding to fenugreek, guar, tara and locust bean, respectively. The fibre diffraction evidence for guar polysaccharide (Palmer & Ballantyne, 1950) and for other polysaccharides with backbones constituted of 1,4 linked β-D-*manno* residues such as mannan (Frei & Preston, 1968), poly-(mannuronic acid) (Atkins, Mackie, Parker & Smolko, 1971), and mannan acetate (Bittiger & Marchessault, 1971), as well as clear indications that have emerged from model-building in the computer (Rees & Scott, 1971), would indicate that any ordered conformation of a galactomannan must involve the backbone in an extended, ribbon-like form.

Gels form when a fairly dilute warm solution (about 1% w/v in water, sometimes less) of agarose or κ-carrageenan is allowed to cool to room temperature. In the presence of a galactomannan, gels may form when agarose or carrageenan is diluted well below the limit at which it would gel alone. Galactomannans do not gel in this way unless mixed with another polysaccharide. The solid-like, stress-resisting bulk properties show that each gel has some type of internal structure. Three alternative and possibly overlapping models could reasonably be suggested from first principles.

(i) The structure results from an aggregation of polysaccharide chains, possibly with a conformation change, to form particles of colloidal dimensions which cohere to form a gel as, for example, in virus gels or microcrystalline cellulose gels.

(ii) The structure results from a phase change or "solidification" of the water, perhaps nucleated or stabilized by polysaccharide chains.

(iii) The structure results from an association of chain segments to form "junction zones" which are joined into a network by chains that run through two or more zones.

The third model is probably correct for most polysaccharide systems (Rees, 1969*a*) and

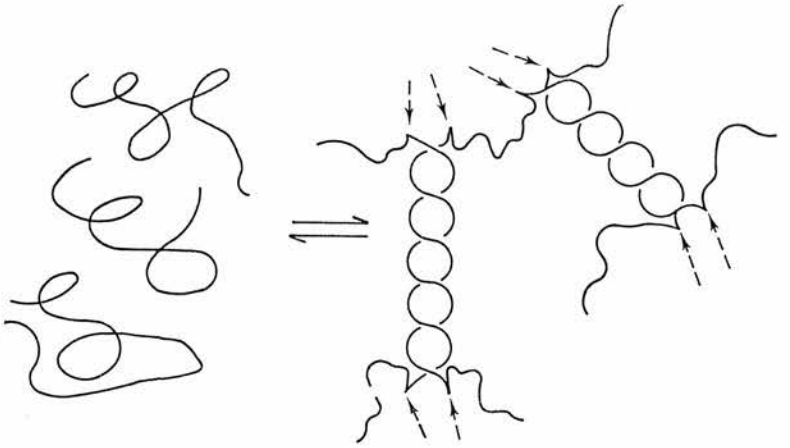


FIG. 1. Model for the molecular basis of gel formation by κ and ι-carrageenan: a network is formed by the combination of chains in double helix formation in a pattern that is influenced by the distribution of the helix-breaking kinks shown by arrows. Especially in κ-carrageenan, the network has extra stability from the aggregation of helices—a feature that isnot shown here.

the junction zones in κ-carrageenan gels probably contain the same helices that are believed to exist in the fibrous state (see above). This model (Fig. 1) is supported by detailed comparison of the development of gel properties and the optical rotation changes attributed to helix formation (Rees, Steele & Williamson, 1969; see also section 5). The gel structure is sensitive to changes in covalent structure in exactly the manner predicted (Rees, 1969*a*) from double helix geometry. We have further suggested that each chain is caused to combine with more than one partner, as is necessary to form a network rather than propagating the same double-helical association along the entire molecular length, by structural irregularities or "kinks" which are helix-terminating. Such kinks have been chemically identified (Anderson, Dolan & Rees, 1968; Rees *et al.*, 1969) and appear to be involved in the biological control of tissue texture (Lawson & Rees, 1970). With a related polysaccharide, ι-carrageenan, selective cleavage at the kinks gave a product which in solution showed an optical rotation shift similar to that which accompanies gel formation even though gel structure does not actually develop (McKinnon, Rees & Williamson, 1969). This result is consistent with model (iii), but is unexpected on the basis of model (i) or model (ii). The product has an average chain length of 30 to 60 disaccharide residues and is shown by osmometry and light-scattering to undergo the dimerization that is required (Fig. 2) by our explanation (R. A. Jones & A. Penman, manuscript in preparation). We have been able to correlate the sign and magnitude of the shift with the details of double helix geometry (Rees, Scott & Williamson, 1970). Finally, several spectroscopic methods (Child, Pryce, Tait & Ablett, 1970; Woessner, Snowden & Chiu, 1970; Woessner & Snowden, 1970; Blandamer & Burdett, 1971) show that the state of the bulk water component does not undergo any large change during melting and liquefaction of polysaccharide gels, thus eliminating model (ii) also.

Thus, these polysaccharide gels owe their structure to the cross-linkage of chains in non-covalent associations and it should be possible to characterize the associations by the properties of the sol–gel interconversion which involves their formation and

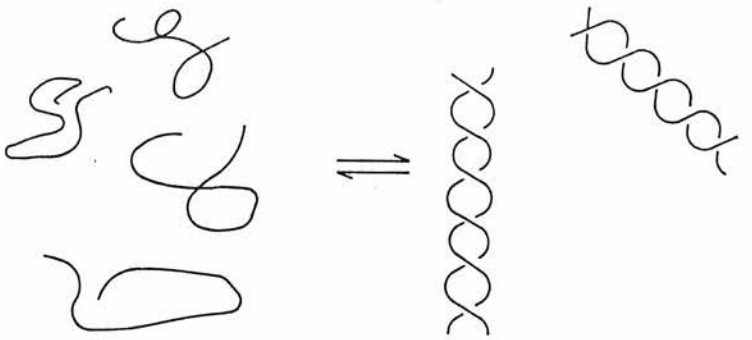


FIG. 2. Proposed behaviour of the segmented κ and ι-carrageenans that are prepared from the parent polysaccharides by selective cleavage of the kinks shown in Fig. 1, when they are heated and cooled in aqueous solution.

destruction. Many experiments are described in this paper which use monochromatic optical rotation to monitor the formation and destruction of these chain-associations, although the shift in optical rotation is actually believed to be caused by the conformation change that occurs as part of the process. This interpretation is supported by detailed investigation of many mono-, di, oligo- and polysaccharides, including quantitative correlations with relevant conformation angles (Whiffen, 1956; Brewster, 1959; Lemieux, Pavia, Martin & Watanabe, 1969; Lemieux & Martin, 1970; Rees, 1970; Rees & Scott, 1971; Rees *et al.*, 1970). Nevertheless, conclusions will also be checked against other methods when possible.

2. Materials and Methods

(a) *Polysaccharide samples*

Two samples of agarose were used with closely similar results. The first was highly purified by chromatography on diethylaminoethyl-Sephadex and was kindly supplied by Dr M. Duckworth (Duckworth & Yaphe, 1971). The second was a production batch of agarose powder (code REX 5468) from Marine Colloids, Inc., Rockland, Maine 04841, U.S.A. Samples from *Gracilaria* species showed rather different behaviour and this will be described elsewhere.

κ-Carrageenan was the same sample as used in earlier work (Rees *et al.*, 1969).

Galactomannans were obtained from several different sources, as explained in the Introduction. Galactomannan A was extracted from seeds of *Trigonella foenum-graecum*, by the method of Andrews, Hough & Jones (1952). The seeds were supplied by Marine Colloids, Inc. Galactomannan B was a purified grade purchased from Kobenhavns Pektinfabrik, Copenhagen, Denmark. Galactomannan C was purified material from Marine Colloids, Inc. (code REX 5922). Various samples of galactomannan D were used, all of which were purchased from Kobenhavns Pektinfabrik, and the results did vary slightly from one sample to another.

It was found that dialysis of polysaccharide samples against distilled water did not affect their behaviour, and, therefore, this step was not normally included in routine experiments. However, all polysaccharides were purified before use by filtration of a hot solution (0·5% w/v or lower concentration) through glass fibre paper and then, while still hot, through a Millipore filter (1·2 or 3 μm pore size) before final freeze-drying. Analysis then showed the following proportions of mannose relative to galactose residues in galactomannans A, B, C and D respectively: 1·08, 1·56, 3·00, 3·35.

(b) *Kink-splitting by the Smith degradation*

This sequence is based on the reactions described by Goldstein, Hay, Lewis & Smith (1965), and an additional reaction (Rees, 1961*a*) for carrageenans only.

Agarose (2 g) was dissolved by autoclaving in water (300 ml.) and then mixed at 40°C with sodium metaperiodate (5·35 g) which had been predissolved in water (200 ml.). Oxidation was allowed to proceed for 75 hr at this temperature before addition of excess of ethylene glycol to halt the reaction, followed by potassium borohydride (6 g in 2·5 l. of water). After 2 days the solution was dialysed against running tap water for 5 days and adjusted to 0·1 N with respect to hydrochloric acid for mild hydrolysis at room temperature for 16 hr. The solution was neutralized with potassium hydroxide and dialysed again for 7 days against running tap water, before evaporation under diminished pressure and final freeze-drying. Yield : 2·17 g.

κ-Carrageenan (25 g) was dissolved in water (4 l.) by boiling for several minutes and then mixed at room temperature with sodium metaperiodate (53·5 g) in water (1 l.). After allowing oxidation to proceed in the dark at room temperature for 65 hr, an excess of ethylene glycol was added. Sodium borohydride (75 g) was dissolved in the solution which was left at 2°C for 72 hr, and then made 1 N with respect to sodium hydroxide before heating for 5 hr at 80°C, with further addition of borohydride (25 g). The mixture was cooled and neutralized with hydrochloric acid and then excess of acid was added to pH 1. After 24 hr at room temperature for the selective hydrolysis step, potassium hydroxide solution was added to neutrality before dialysis against running tap water for 6 days, in the presence of thymol as preservative. The solution was dialysed against daily changes of distilled water for 6 days and then passed through a column of Amberlite IR120 ion exchange resin (K^+ form) and evaporated under diminished pressure to 6 l. Potassium chloride solution (2 M; 240 ml.) was then added followed by isopropanol (480 ml.). After warming to 70°C, the solution was cooled slowly and filtered. The precipitate, which represented 32·5% of the total carbohydrate according to assay with the phenol/sulphuric acid reagents (Dubois, Gilles, Hamilton, Rebers & Smith, 1956) was collected on the centrifuge and dissolved in water for dialysis against running tap water for 7 days and then against daily changes of distilled water for a further 7 days. After passage through an ion exchange resin (IR 120, K^+ form), the solution was freeze-dried.

To the supernatant solution was added isopropanol (420 ml.), which precipitated a further 30% of the polysaccharide. The product was worked up in the same way as the previous fraction and used as "segmented κ-carrageenan" in the experiments described below.

(c) *Spectroscopic methods*

Polysaccharide systems were prepared by dispersion in water, followed by sealing and autoclaving (15 lb/in^2) for about 25 min. To ensure that there was no interference from dust, all solutions were passed hot through a Millipore filter (1·2 μm pore size for carrageenan systems, 0·45 μm for agarose) into hot jacketed cells.

Optical rotation measurements were with the Perkin-Elmer 141 polarimeter using 1-cm cells for carrageenan systems and 10-cm cells for agarose, at wavelengths of 365, 436, 546 and 589 nm. Although all systems showed plain dispersion curves, the use of several wavelengths did have advantages: readings were amplified at 365 and 436 nm (useful for dilute systems), whereas the high light intensities at 436 and 546 nm were useful when transmission was poor, and measurements at 589 nm could conveniently be compared with values in the literature. Care was taken to allow readings to become steady before making each measurement; for agarose/galactomannan systems in the transition region, this required many hours and equilibration was normally overnight. Temperature control was by circulating water baths with contact thermometers which were set manually. Apparent variations in optical rotation which arose from temperature-induced cell strain were measured with the cell containing distilled water, and appropriate corrections were made. Refractive index and density variations were shown to be small and were, therefore, neglected.

Ultraviolet spectra were recorded with the Unicam SP800 spectrophotometer, using a path length of 1 cm and a standard cell holder which allowed water circulation from the thermostat bath. Samples were thoroughly dialysed to avoid interference by salts and

clarified with Teflon Millipore filters (pore size 5 μm). The usual cellulose acetate filters were not used because they were found to retain the methylene blue that was present in these experiments.

Circular dichroism spectra were recorded with the Cary 61 spectropolarimeter and a path length of 0·1 cm, with methods that were otherwise identical to those for ultraviolet spectroscopy.

(d) *Determination of gel points*

To determine the setting temperature, the sol was cooled in the appropriate range in steps of 2 deg. C with equilibration for about 30 min at each stage. Glass beads (1·3 mm $\pm$ 0·1 mm diameter) were then introduced at the surface or just below and the setting temperature was taken to be the stage at which these failed to sink.

To determine the liquefaction temperature, the gel was allowed to age below its setting temperature for at least a few hr, with glass beads that remained suspended in it from the setting point determination. It was then heated through the appropriate range in steps of 2 deg. C with equilibration as before, and the liquefaction temperature was taken as the stage at which the beads sank.

(e) *Analyses of galactomannans*

These were kindly done by Dr A. Morrison of this Laboratory, by gas chromatography of the mixture of galactitol and mannitol hexa-acetates that resulted from hydrolysis, reduction and acetylation.

(f) *Freeze–thaw experiments*

Fehlings solution was used for recovery of galactomannans, in the same way as in the preparation of galactomannan A (Andrews *et al.*, 1952). The polysaccharide contents of fluids which drained away when frozen gels were thawed, were estimated by the phenol/sulphuric acid method (Dubois *et al.*, 1956).

(g) *X-ray diffraction*

Methods were as described elsewhere (Anderson *et al.*, 1969), using a closed camera with a lead glass collimator which was constantly flushed with helium at a controlled humidity.

(h) *Computer model-building*

These calculations were based on the methods and assumptions of Rees & Scott (1971).

3. Results for Single-polysaccharide Systems

It was found that κ-carrageenan could be converted by the "kink-splitting" reaction sequence that had been used earlier for ι-carrageenan (McKinnon *et al.*, 1969) to a product which showed a sharp shift in optical rotation when heated or cooled in solution but did not gel. This short-chain product will be known as segmented κ-carrageenan. When compared with the native polysaccharide at the same concentration (Fig. 3), the optical rotation shifts start at about the same temperature on the cooling curve and follow the same course before they diverge progressively and at room temperature the relative shifts suggest a helix content of 50 to 70% for the gel relative to the segments. The re-heating curves show that helix melting is relatively delayed in the gel (Fig. 3). Hysteresis is observed for both systems (Fig. 3) but only for the first part of the transition. Optical rotation changes occur with setting and liquefaction of agarose gels, as shown in Figure 4. They differ from carrageenan behaviour only in that the signs of the shifts are reversed and the hysteresis is larger.

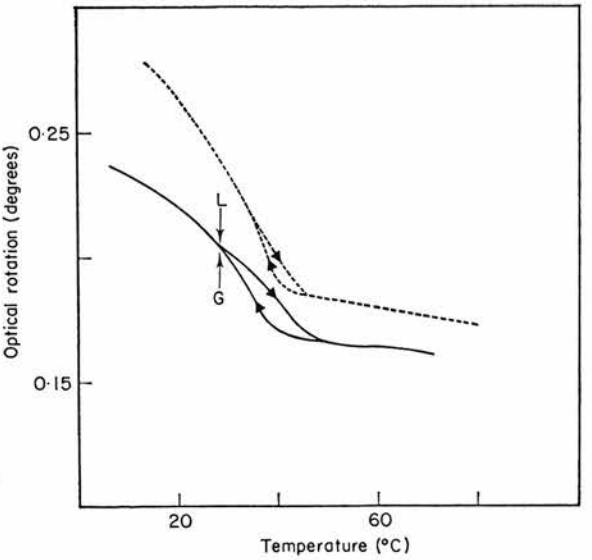


FIG. 3. Comparison of the optical rotation variations with temperature for native κ-carrageenan (———) and segmented κ-carrageenan (– – – – –). Heating and cooling curves are distinguished by the arrows. Measurements were at 546 nm with 3% w/v solutions. The gel point (G) and liquefaction point (L) are shown by the arrows.

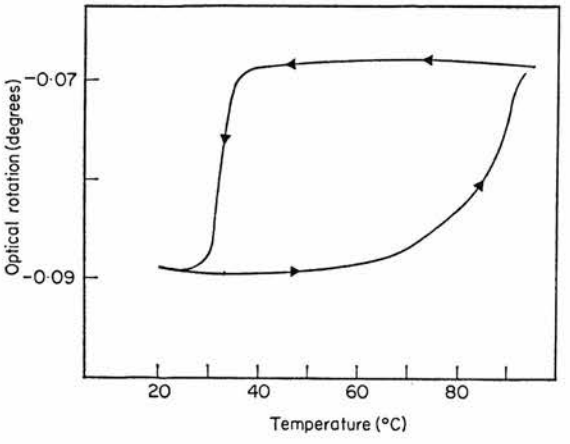


FIG. 4. Optical rotation changes during the setting and liquefaction of an agarose gel (0·2%). Heating and cooling curves are distinguished by the arrows. Measurements were at 546 nm.

None of the galactomannans, when heated and cooled in aqueous solution, gave an optical rotation change to suggest a conformation transition. A typical curve is shown in Figure 5.

4. Results for Interacting Systems

It was already known that a solution of native (undegraded) κ-carrageenan, when diluted until it would not form a gel when cooled alone, could gel if mixed with

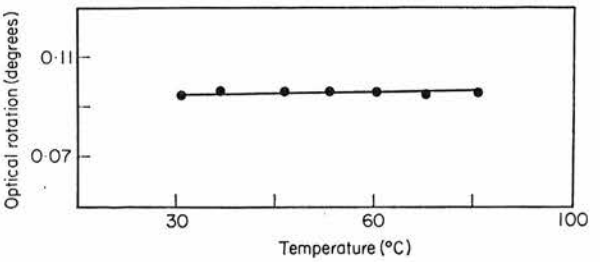


FIG. 5. Variation of optical rotation of galactomannan D with temperature. Measurements were at 546 nm with a 1% solution in a 10-cm cell. Galactomannans A, B and C showed similar behaviour.

galactomannan D. We have now discovered that segmented κ-carrageenan—which would not gel alone at any concentration—also forms firm rubbery gels in mixtures with galactomannan D. Both effects diminish from one galactomannan to another in the direction of increasing galactose content (Table 1). Indeed, the galactomannans having highest contents of galactose residues did not show the interaction at all.

TABLE 1

Gel formation in mixed systems containing galactomannans

Galactomannan used	Minimum concentration of galactomannan (% w/v) which is effective in forming a gel structure to support 1·3 mm glass beads when mixed with:		
	Segmented κ-carrageenan (K^+ salt, 2% w/v)	κ-carrageenan (K^+ salt, 1% w/v)	agarose† (0·05% w/v)
Galactomannan A	No gel	No gel	1
Galactomannan B	No gel	No gel	0·7
Galactomannan C	3	3	0·1
Galactomannan D	1	1	0·05

† These results are for native agarose; segmented agarose at 0·2% concentration was gelled by 1% galactomannan D but no attempt was made to examine other mixtures which contained these segments.

When heated and cooled, the mixtures with galactomannan D showed optical rotation changes, including partial hysteresis, of the type normally seen in carrageenan systems (Fig. 6). Although as usual, liquefaction occurs at a different temperature from gel setting, both these bulk changes correspond to a much lower conversion to helix than they do in the absence of galactomannan (compare Figs 3 and 6). More careful comparison of the optical rotation loops for segmented κ-carrageenan in the presence and absence of galactomannan D shows other significant differences in detail: the loop is broadened by the presence of galactomannan and shifted to higher temperature (Fig. 7). Despite this distinct change, comparison of loops in terms of the first

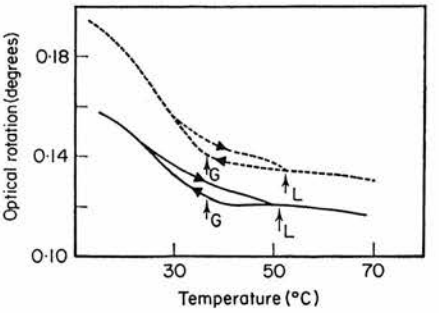


FIG. 6. Comparison of the optical rotation variations with temperature for native κ-carrageenan (———) and segmented κ-carrageenan (– – – – –) in the presence of galactomannan D. Heating and cooling curves are distinguished by the arrows. Measurements were at 546 nm with solutions that were 1% with respect to galactomannan and 2% with respect to carrageenan. The gel point (G) and liquefaction point (L) are shown.

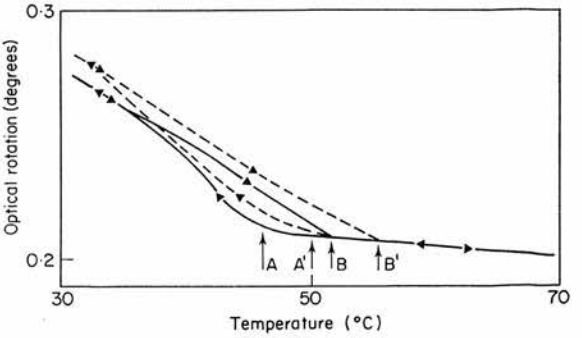


FIG. 7. Comparison of the optical rotation variations with temperature for segmented κ-carrageenan (4%) (———) and a mixture of segmented κ-carrageenan (4%) and galactomannan D 1% (– – – – –), the former curve being corrected by addition of the contribution from galactomannan D when run separately (compare Fig. 5). Points A, A′, B, B′ mark the inflections that are discussed in the text and in the caption to Fig. 8.

inflection on each cooling curve (points A and A′ on Fig. 7) and the last inflection on each heating curve (points B and B′ on Fig. 7) shows that the influence of κ-carrageenan concentration has roughly the same form whether galactomannan is present or not (Fig. 8).

Methylene blue is a known conformational probe for carrageenans (D. A. Rees & F. B. Williamson, unpublished work). In a hot solution of native or segmented κ-carrageenan in the random coil form, this dye shows its normal ultraviolet spectrum and no circular dichroism spectrum (Fig. 9). With cooling to permit conversion to helix, however, there is a shift in the ultraviolet peak and a characteristic optical activity is induced in the circular dichroism spectrum (Fig. 9). These spectra and spectral changes were also observed when galactomannan D was added.

When agarose was subjected to the kink-splitting sequence of reactions that had been used with carrageenans, the product had again lost the ability to gel. Instead, when the solution was cooled to the temperature range for gelation of the native polysaccharide, a powdery precipitate formed which dissolved on reheating. As with

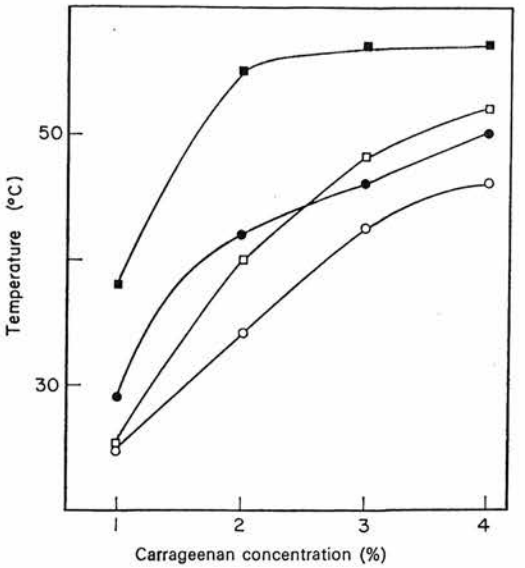


FIG. 8. Variation of temperature of inflection (see Fig. 7) with polysaccharide composition. —○—○—, Cooling inflection for segmented κ-carrageenan alone; —●—●—, cooling inflection for segmented κ-carrageenan mixed with galactomannan D (1%); —□—□—, heating inflection for segmented κ-carrageenan alone; —■—■—, heating inflection for segmented κ-carrageenan mixed with galactomannan D (1%).

κ-carrageenan, the segmented product formed a firm rubbery gel in mixtures with galactomannan D. Hysteresis was observed in the melting and setting of this mixed gel.

As an example of the interaction of native (undegraded) agarose with galactomannan D, a particular preparation of agarose did not gel alone in aqueous systems at less than 0·1% concentration and likewise a 2% solution of galactomannan D did not gel: however, when both polysaccharides were present together, a distinct gel structure could be formed with 2% galactomannan and levels of agarose as low as 0·01%. As with κ-carrageenan, the effect became less marked with increasing galactose content of the galactomannan, as shown in Table 1. In another experiment, a series of gels was prepared, each containing 0·5% agarose and 0·1% of a different galactomannan. When frozen then thawed, each gel shrank and released about half the volume as fluid in the normal way for agarose gels. Analysis showed that the mixed gel prepared with galactomannan D released only about one-tenth of the galactomannan in this fluid. If the fluid retained in the gel had roughly the same composition as that released, then it follows that 80% of this galactomannan cannot be accounted for unless it is bound into the network. Progressively less was bound of the other galactomannans in order of increasing galactose content, thus: 70% of galactomannan C, 27% of galactomannan B and 17% of galactomannan A. When the shrunk gel which remained after freeze–thaw treatment was dissolved in warm water, galactomannan could be precipitated with Fehling's solution (a standard galactomannan precipitant). However, if the solution was cooled almost to gel point before addition of Fehling's solution, no precipitate was seen. Analysis of this recovered galactomannan showed that the ratio of galactose : mannose residues was 1 : 4·25 compared with the value of 1 : 3·35 before preparation of the gel for freezing.

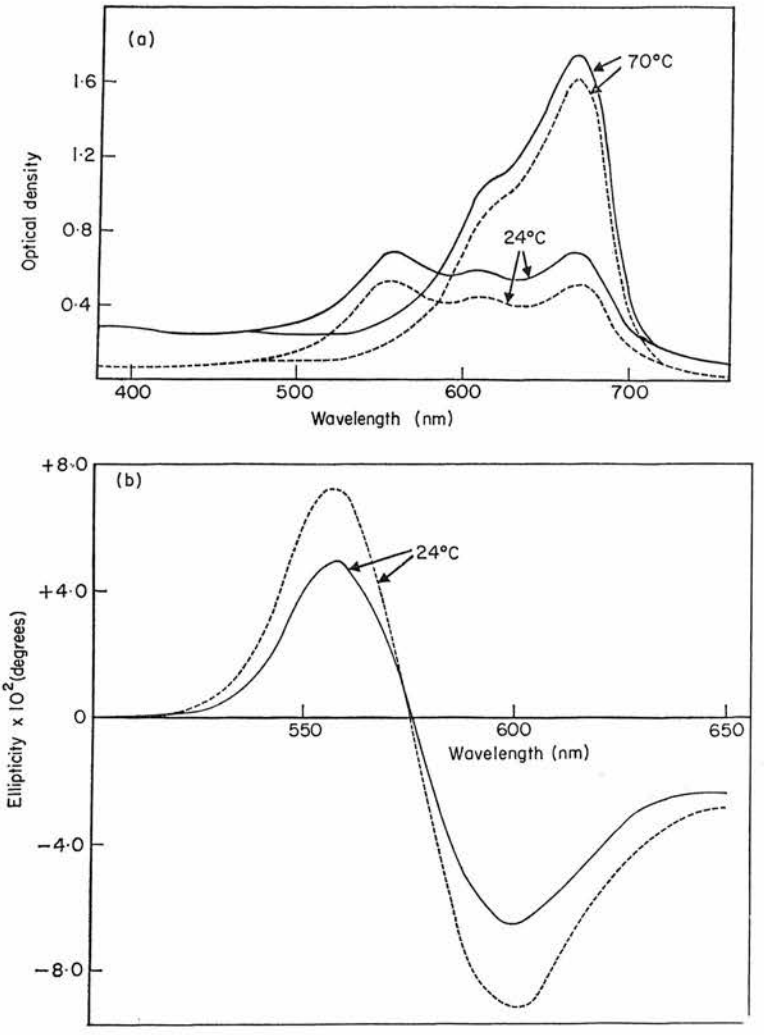


FIG. 9. Spectra recorded for methylene blue (9·8 mg/l.) in the presence of segmented κ-carrageenan (2%) (———) and a mixture of segmented κ-carrageenan (2%) with galactomannan D (1%) (– – – – –). Curves are labelled to show the temperature at which each spectrum was recorded. Ultraviolet spectra (a) show the appearance of a peak at 558 nm when the solution is cooled; this is attributed to helix-bound dye. Circular dichroism spectra (b) show the Cotton effects at low temperature only: at high temperature the spectrum coincides with the base-line.

Both in the absence and presence (Fig. 10) of galactomannan D there is a sharp shift in the optical rotation of an agarose solution when it is cooled to about 35°C and gelation begins. However, the shifts are opposite in sign (Fig. 10); the negative shift of agarose becomes increasingly positive as more galactomannan is added until a limit is reached (Fig. 11). Reheating also shows differences, especially that galactomannan causes the curve to cross the cooling curve to give a butterfly shaped hysteresis form instead of the usual loop (Fig. 10).

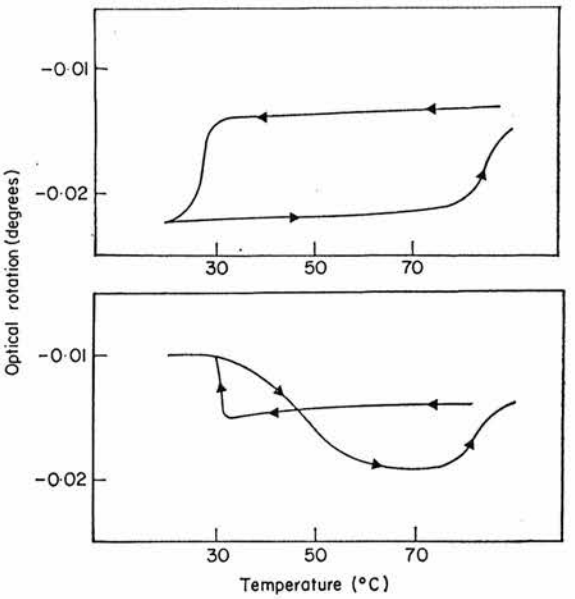


FIG. 10. Comparison of the optical rotation variations with temperature (above) for agarose at a non-gelling concentration (0·05%) and (below) for a gelling mixture of agarose (0·05%) and galactomannan D (0·1%). To facilitate comparison, the lower curve has been adjusted by subtracting the contribution expected from galactomannan alone (compare Fig. 5). Note the similarity in behaviour of non-gelling (upper curve) and gelling (Fig. 4) concentrations of agarose, indicating the same conformation change, although the transition temperature shifts slightly with concentration.

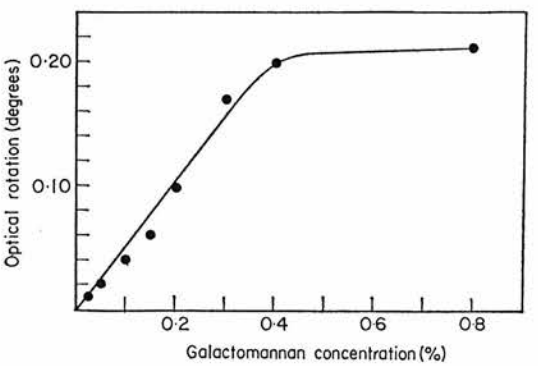


FIG. 11. Variation of optical rotation shift in mixtures of agarose (0·05%) and galactomannan D. The shift is relative to the optical rotation of each mixture at high temperature. Measurements were at 589 nm.

The magnitude of the positive shift depends on agarose as well as galactomannan concentration: when the agarose concentration in mixtures with 0·5% galactomannan D was increased from 0·025 through 0·05 to 0·12%, the optical rotation shifts at 436 nm changed from 0·030 to 0·060 to 0·075°. (These values are corrected for a predicted variation in agarose contribution.)

5. Interpretation

Experiments with the systems that contained carrageenan alone confirmed that, as previously suspected, κ-carrageenan chains resemble ι-carrageenan (see Introduction and Figs 1 and 2) in having sections with helix-forming potential that can be split out by chemical reaction to give a product which has lost the ability to gel.

Segmented κ-carrageenan was very similar to native κ-carrageenan in all its behaviour except that it did not form gels. The minor differences that did exist in the variation of optical rotation with temperature were of a kind that could be explained by network influences on setting and melting of helices in the gelling system, and perhaps by loss in the dialysis step of those chain parts that were relatively rich in kinks. Both systems showed hysteresis (Fig. 3), for which the explanation (Rees *et al.*, 1969) is that the helix does not form as soon as it would be thermodynamically stable on the cooling curve (i.e. at its melting temperature) because nucleation is rate-limiting. It is not known whether the critical nucleus lies on the path to the individual helix or to a helix aggregate, but cooling below the melting temperature is evidently required to diminish its free energy sufficiently to allow the transition to occur. However, the chief importance of hysteresis here is not in the details of its explanation but in the fingerprint that it provides, with the sigmoidal heating and cooling curves, for the helix-coil transition of κ-carrageenan.

The specific optical rotation of agarose at high temperature (—28° at the sodium D line) is roughly that expected (—22°) for the random coil, based on optical rotations of the component disaccharides and on the assumption of an idealized covalent structure (compare Rees, 1969*a*; Duckworth *et al.*, 1971). According to our empirical method for correlating optical activity with conformation (Rees, 1970; Rees *et al.*, 1970; Rees & Scott, 1971), the value at low temperature (—44°) would correspond to a conformation for which the sum of the sines of the four conformation angles, measured from appropriate origins, is at least 1·8 and is indeed rather greater if conformation interconversion in the gel is incomplete. This cannot be compared directly with a known conformation for the solid state because X-ray diffraction photographs for agarose are (so far) very disappointing. However, they do show four layer lines with spacings that correspond to a fibre axis translation of 9·5 Å ± 0·3 Å and we therefore used computer model-building to search for conformations that are sterically possible and simultaneously compatible with this diffraction evidence and with the optical rotation shift. The most likely possibilities on steric grounds were double helices with three or four disaccharide residues per left-handed turn; in terms of $\Delta\phi^{AB}$, $\Delta\psi^{AB}$, $\Delta\phi^{BA}$ and $\Delta\psi^{BA}$ (Rees, 1970; Anderson *et al.*, 1969), these occurred around (60, 20, 10, 50°).

The multiply-stranded conformations are attractive because gel formation must involve chain-association, and such helices are already known to provide this in gelatin and carrageenan (Rees, 1969*a*). The pronounced chemical and physical similarity of agarose to gelling carrageenans has already suggested that the conformation might be carrageenan-like (Rees, 1969*a*,*b*). Agarose can be regarded as a diastereomeric derivative of κ- or ι-carrageenan because it contains residues of 3,6-anhydro-α-L-galactose instead of the D-enantiomer; if it forms a double helix, it is interesting that the change of residue configuration reverses the screw sense (since all possibilities had left-handed chains) as well as the direction of optical rotation shift. Although we believe that κ-carrageenan and agarose each exist in an ordered conformation at

low temperatures in solution, we emphasize that the character of this conformation is known only for κ-carrageenan. For simplicity, both conformations will be referred to as 'helices' and general schemes will be drawn in terms of the carrageenan double helix.

As with carrageenans, Smith degradation of agarose led to a product which underwent the conformation change without gel formation. There has previously been no evidence that periodate-oxidizable kinking residues are present in agarose, although their presence is to be expected on biosynthetic grounds (Rees, 1961*b*) as well as by analogy with carrageenans. Only the very first stages of the transition could be observed by optical rotation because the segmented polysaccharide then separated rapidly from solution. Such behaviour is to be expected (Flory, 1956) for a chain molecule which is not solubilized by ionic substituents such as occur in carrageenans, when it becomes rigid and hence loses conformational entropy.

The ability of certain galactomannans to form gel structures when mixed with other non-gelling polysaccharides (i.e. with segmented κ-carrageenan or segmented agarose) or with gelling polysaccharides at concentrations that would otherwise be too dilute (native κ-carrageenan or native agarose) would imply a network that forms by interaction of unlike chains, perhaps by a mutual binding. Evidence cited in the Introduction would show that any ordered conformation of the galactomannan would be fairly fully extended and alternately rather densely and rather lightly substituted with galactose residues; hence, if ever the random coil converts to an ordered form—for example in binding to another chain—this can be represented as a molecular ribbon that is alternately "hairy" and "smooth" (Fig. 12). The mixed gels do indeed show sharp

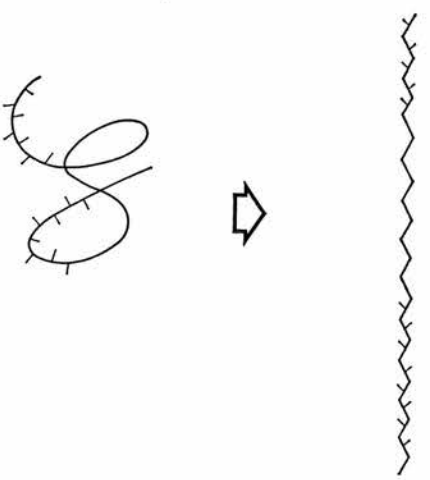


FIG. 12. Two conformations of galactomannan and their hypothetical interconversion. Left, random coil; right, ribbon-like form. In the representation of the galactomannan, galactose residues are shown as protrusions from the continuous mannan backbone. Although this Figure shows only one smooth and two hairy sequences in the chain, a galactomannan may actually contain many such sequences in alternation.

melting and setting behaviour consistent with co-operative transitions and hence with networks that are cross-linked by ordered, non-covalent associations. Comparison of the gelling action of galactomannans (Table 1 and other evidence) would indicate that

the smooth sites are the more effective in any binding. On the other hand, carrageenan chains probably enter the cross-links in their helical form because the characteristic optical rotation behaviour and its concentration dependence, would suggest that network formation is coupled to the helix $\rightleftharpoons$ coil transition, and the methylene blue probe confirms that helices are formed in the mixtures with galactomannan. All the arguments in this paragraph therefore point to the working hypothesis that ordered binding occurs between the ribbon-like smooth sites on the galactomannan chains and the helical carrageenan or agarose; this is shown schematically in Figure 13. If, in this

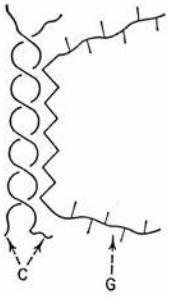


FIG. 13. Model proposed for interaction between chains of κ-carrageenan (C) and galactomannan (G). For explanation of the representation of galactomannan, see the legend to Fig. 12.

way, the segmented helices (Fig. 2) bind more than one smooth galactomannan site, the cross-links that were split by chemical reaction are thereby restored. Similarly, when native agarose or native κ-carrageenan is too dilute to form a continuous network throughout the entire solution, the binding could cause galactomannan chains to be incorporated and provide extension.

This model is confirmed by more subtle features of optical rotation behaviour. For example, the helix $\rightleftharpoons$ coil transition for κ-carrageenan is evidently perturbed by the presence of galactomannan (Figs 7 and 8), pointing to an interaction effect. The shift of the cooling transition shows that the critical nucleus for helix formation corresponds to a lower free energy in the presence of galactomannan, as could be caused by spontaneous non-covalent and ordered binding of galactomannan to the growing helix, i.e. ligand-induced formation of the nucleus (compare Koshland, 1971). The effect on helix melting is substantially larger (Figs 7 and 8) and is likewise consistent with stabilization by binding.

The perturbation of the optical rotation behaviour of agarose is very different but is easily interpreted in terms of the same type of interaction. Gelation occurs at about the temperature that agarose is expected to convert to its ordered tertiary structure, as expected if the binding of galactomannan requires agarose in this form. However, as for carrageenan and presumably for the same reason, the transition is actually "induced" at a slightly higher temperature. The direction of the optical rotation shift suggests a conformation change of the galactomannan which makes a large positive contribution to override the usual negative contribution from agarose. This interpretation is spectacularly confirmed by the behaviour during reheating when, owing to the enormous hysteresis which is a property of the agarose helix, this form survives while galactomannan/agarose associations "melt" with loss of the positive contribu-

tion (Fig. 10). Despite other differences, the two heating curves on Figure 10 then correspond closely above 70°C in both shape and relation to the cooling curve, as required for an internally consistent explanation. The details of optical rotation behaviour depend on the concentrations of both polysaccharides and there is evidence for a saturation effect in the amount of galactomannan that can be bound by agarose (Fig. 11), all of which is expected for an interaction effect.

When the variation of the optical rotation shift in agarose/galactomannan mixtures (Fig. 11) is used to calculate the effective change in specific optical rotation of galactomannan (Fig. 14) it becomes apparent that a progressively higher proportion of

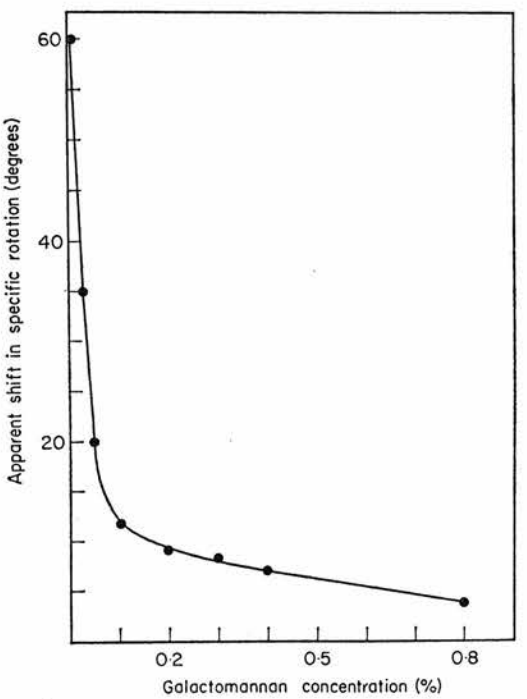


FIG. 14. Variation of optical rotation shift in mixtures of agarose (0·05%) and galactomannan D. The shift is expressed as an apparent shift in the specific optical rotation of galactomannan and is calculated by taking into account the contribution expected from the agarose component alone.

mannose residues is bound as the galactomannan concentration diminishes. This follows because their average contribution to the optical rotation shift increases and is to be expected if agarose has a finite number of interaction sites for which galactomannan chains compete. At the lowest galactomannan concentration which allows measurement, the shift was 180 $\pm$ 30° in molecular rotation per unsubstituted mannose residue. The starting molecular rotation of these smooth regions before they were bound to agarose is found by extrapolation of the homologous oligosaccharides (Schwarz & Timell, 1963), giving −87° per mannose residue. Thus the absolute molecular rotation of the bound conformation is at least 63° per smooth mannose residue and could be much greater. In the usual way (Rees, 1970), this is found to correspond to a conformation for which the sum of the sines of the conformation

angles (as $\Delta\phi$ and $\Delta\psi$; Rees, 1970) is $-0{\cdot}44$, or even more negative. Since this conformation is supposed to bind in an ordered way to an agarose helix which has a pitch of about 9·5 Å, it must have a repeat period that corresponds to this value or a simple multiple of it. Calculations show a right-handed threefold conformation, two periods of which could match with three of agarose and which would correspond to $\sin\Delta\phi+\sin\Delta\psi \approx -1{\cdot}4$.

This mannan conformation provides a self-consistent explanation of the optical rotation changes in semi-quantitative terms, so consolidating the qualitative explanation in preceding paragraphs.

Further confirmation of a binding of agarose to galactomannan that occurs at the gel point, was that galactomannan was protected in mixtures at the gelation threshold against its usual complexation with cuprammonium but was not protected at higher temperatures when the association is expected from our other evidence to be reversed. Analysis of the interstitial fluid that is released from the gel by a freeze–thaw treatment also gave evidence for binding to the network of galactomannan chains having smooth sites.

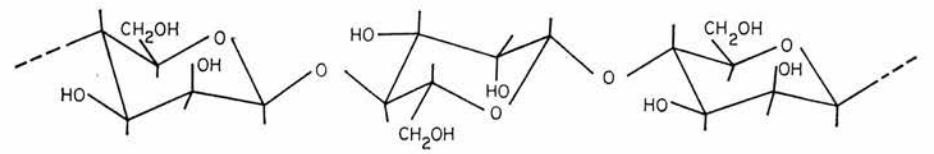
6. Final Discussion and Conclusions

Because of the novelty of the phenomena which we report in this paper, there are no standard rigorous experimental methods available to characterise them, and our work has had to be based on background knowledge in which there are many gaps. This is why our arguments are roundabout and unorthodox, even though we would claim that, ultimately, the conclusions are firmly established that (i) association exists between carrageenan double helices and sequences of unsubstituted mannose residues in the galactomannan and (ii) there is a broadly similar interaction involving the agarose tertiary structure. These associations are non-covalent and ordered but we know nothing about their geometry other than that which is implicit in the geometry of the carrageenan double helix itself and in the steric restrictions which constrain the mannan backbone to a ribbon-like shape. Neither do we know yet the "stoichiometry" of the association (the number of galactomannan-binding sites per helix, or helix-binding sites per galactomannan segment), or the length of helix involved in each association, or indeed the polymerisation state of the associated form (whether each helix can exist independently with its complement of galactomannan(s), or whether binding only occurs within some multiple of this unit). However, the conclusion is inescapable that some association must exist of the type shown schematically in Figure 13, even if it is only as part of more complex assemblies.

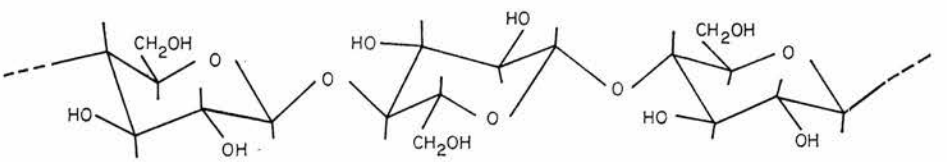
Non-reserve polysaccharides seem to function in biological tissues through the part they play in the cohesion, the retention of water and salts, the physical organization, and the elasticity and general texture. Such properties are obviously determined by conformation and association as well as molecular constitution. The role of conformation and of association between like chains (i.e. the role of secondary and tertiary structure) has begun to be understood recently (Rees, 1969*a*). The systems described in this paper now provide models for the association between different polysaccharide entities, i.e. for the quaternary structure. We use the protein nomenclature to underline that polysaccharides, like most biological chain-molecules, have a propensity of interacting co-operatively when they perform their roles. A further analogy with protein behaviour is that, in combination with galactomannan, the carrageenan helix can be caused to form or persist when it would otherwise be unfavourable (Fig. 7); this

influence is, therefore, an example of ligand induction of polysaccharide conformation (compare Koshland 1971).

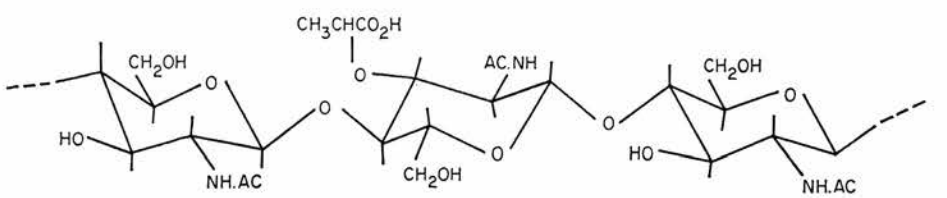
The smooth regions of galactomannan (formula IV) are similar in structure to important skeletal polysaccharides such as hemicelluloses, cellulose (V), and peptidoglycan (VI), suggesting that associations with agarose and κ-carrageenan occur in



IV



V



VI

IV–VI Comparison of the backbone structures of mannan (IV), cellulose (V) and peptidoglycan (VI).

imitation of natural associations between the latter polysaccharides and flexible chains bound in the microfibrillar structure with which they co-exist. In another system (E. R. Morris & D. A. Rees, unpublished work) we have observed association between galactomannan and a bacterial polysaccharide which, in the natural state, presumably exists with peptidoglycan.

It is unusual for conformation transitions to occur in polysaccharide solutions (Rees, 1967) and for the biological associations of polysaccharides to be readily and conveniently temperature-reversible. We have been fortunate to discover systems in which both types of behaviour are modelled and can be observed together or (to a degree) separately. It is expected that they will be useful for further development of the theory of conformation and of biological function.

We thank Mr Ralph Moorhouse for some of the experiments, Dr E. R. Morris for discussion and advice, and Marine Colloids, Inc. for a research grant to support the first stages of this work at the University of Edinburgh.

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